METHOD OF USING MODIFIED OLIGONUCLEOTIDES FOR HEPATIC DELIVERY

FIELD OF THE INVENTION

This application is directed to oligonucleotides and oligonucleosides functionalized to include lipophilic moieties. Relative to their unfunctionalized parent compounds, such lipophilic oligonucleotide conjugates have improved biostability and altered biodistribution in mammals. In one embodiment, such lipophilic oligonucleotide conjugates are used in a method of targeting antisense oligonucleotides to hepatic tissues and thereby preferentially modulating gene expression in the liver and associated tissues of a mammal.

BACKGROUND OF THE INVENTION

Messenger RNA (mRNA) directs protein synthesis. Antisense methodology is the complementary hybridization of relatively short oligonucleotides to mRNA or DNA such that the normal, essential functions of these intracellular nucleic acids are disrupted. Hybridization is the sequence-specific hydrogen bonding via Watson-Crick base pairs of oligonucleotides to RNA or single-stranded DNA. Such base pairs are said to be complementary to one another.

The naturally occurring events that provide the disruption of the nucleic acid function, discussed by Cohen in *Oligonucleotides: Antisense Inhibitors of Gene Expression*, CRC Press, Inc., Boca Raton, Florida (1989) are thought to be of two types. The first, hybridization arrest, denotes the terminating event in which the

oligonucleotide inhibitor binds to the target nucleic acid and thus prevents, by simple steric hindrance, the binding of essential proteins, most often ribosomes, to the nucleic acid. Methyl phosphonate oligonucleotides (Miller, *et al.*, *Anti-Cancer Drug Design*, 1987, *2*, 117) and α-anomer oligonucleotides are examples of antisense agents that are thought to disrupt nucleic acid function by hybridization arrest.

The second type of terminating event for antisense oligonucleotides involves the enzymatic cleavage of the targeted RNA by intracellular RNase H. A 2'-deoxyribofuranosyl oligonucleotide or oligonucleotide analog hybridizes with the targeted RNA and this duplex activates the RNase H enzyme to cleave the RNA strand, thus destroying the normal function of the RNA. Phosphorothioate oligonucleotides are the most prominent example of an antisense agent that operates by this type of antisense terminating event.

Considerable research is being directed to the application of oligonucleotides and oligonucleotide analogs as antisense agents for diagnostics, research reagents and potential therapeutic purposes. At least for therapeutic purposes, and for research purposes involving whole cells, tissues or animals, the antisense oligonucleotides and oligonucleotide analogs must be transported across cell membranes or otherwise taken up by cells in order to exhibit activity. One method for generally increasing membrane or cellular transport is by the attachment of a pendant lipophilic group. More specifically, Ramirez *et al.* (*J. Am. Chem. Soc.*, 1982, *104*, 5483) introduced the phospholipid group 5'-O-(1,2-di-O-myristoyl-sn-glycero-3-phosphoryl) into the dimer TpT independently at the 3' and 5' positions.

Subsequently Shea *et al.* (*Nuc. Acids Res.*, 1990, *18*, 3777) disclosed oligonucleotides having a 1,2-di-O-hexyldecyl-rac-glycerol group linked to a 5'-phosphate on the 5'-terminus of the oligonucleotide. Certain of the Shea *et. al.* authors also disclosed these and other compounds in patent application PCT/US90/01002. A further glucosyl phospholipid was disclosed by Guerra *et al.* (*Tetrahedron Letters*, 1987, *28*, 3581).

In other work, a cholesteryl group was attached to the inter-nucleotide linkage between the first and second nucleotides (from the 3' terminus) of an oligonucleotide. This work is disclosed in United States Patent No. 4,958,013 and further by Letsinger *et al.* (*Proc. Natl. Acad. Sci. USA*, 1989, *86*, 6553). The aromatic intercalating agent anthraquinone was attached to the 2' position of a sugar fragment of an oligonucleotide as reported by Yamana *et al.* (*Bioconjugate Chem.*, 1990, *1*, 319). The same researchers placed pyrene-1-methyl at the 2' position of a sugar (Yamana *et. al.*, *Tetrahedron Lett.*, 1991, *32*, 6347).

Lemairte *et al.* (*Proc. Natl. Acad. Sci. USA*, 1986, *84*, 648) and Leonetti *et al.* (*Bioconjugate Chem.*, 1990, *1*, 149). The 3' terminus of the oligonucleotides each include a 3'-terminal ribose sugar moiety. The poly(L-lysine) was linked to the oligonucleotide via periodate oxidation of this terminal ribose followed by reduction and coupling through a N-morpholine ring. Oligonucleotide-poly(L-lysine) conjugates are described in European Patent application 87109348.0. In this instance the lysine residue was coupled to a 5' or 3' phosphate of the 5' or 3' terminal nucleotide of the oligonucleotide. A disulfide linkage has also been utilized at the 3' terminus of an oligonucleotide to link

a peptide to the oligonucleotide (Corey *et al.*, *Science*, 1987, *238*, 1401; Zuckermann, *et al.*, *J. Am. Chem. Soc.*, 1988, *110*, 1614; and Corey *et al.*, *J. Am. Chem. Soc.*, 1989, *111*, 8524).

Nelson *et al.* (*Nuc. Acids Res.*, 1989, *17*, 7187) describe a linking reagent for attaching biotin to the 3'-terminus of an oligonucleotide. This reagent, N-Fmoc-O-DMT-3-amino-1,2-propanediol is now commercially available from Clontech Laboratories (Palo Alto, CA) under the name 3'-Amine on. It is also commercially available under the name 3'-Amino-Modifier reagent from Glen Research Corporation (Sterling, VA). This reagent was also utilized to link a peptide to an oligonucleotide as reported by Judy *et al.* (*Tetrahedron Letters*, 1991, *32*, 879). A similar commercial reagent (actually a series of such linkers having various lengths of polymethylene connectors) for linking to the 5'-terminus of an oligonucleotide is 5'-Amino-Modifier C6. These reagents are available from Glen Research Corporation (Sterling, VA). These compounds or similar ones were utilized by Krieg *et al.* (*Antisense Research and Development*, 1991, *1*, 161) to link fluorescein to the 5'-terminus of an oligonucleotide. Other compounds of interest have also been linked to the 3'-terminus of an oligonucleotide. Asseline *et al.* (*Proc. Natl. Acad. Sci. USA*, 1984, *81*, 3297) described linking acridine on the 3'-terminal phosphate group of an poly (Tp) oligonucleotide via a polymethylene linkage. Haralambidis *et al.* (*Tetrahedron Letters*, 1987, *28*, 5199) report building a peptide on a solid state support and then linking an oligonucleotide to that peptide via the 3' hydroxyl group of the 3' terminal nucleotide of the oligonucleotide. Chollet (*Nucleosides & Nucleotides*, 1990, *9*, 957)

attached an Aminolink 2 (Applied Biosystems, Foster City, CA) to the 5' terminal phosphate of an oligonucleotide. Chollet then used the bifunctional linking group SMPB (Pierce Chemical Co., Rockford, IL) to link an interleukin protein to the oligonucleotide.

An EDTA iron complex has been linked to the 5 position of a pyrimidine nucleoside as reported by Dreyer *et al.* (*Proc. Natl. Acad. Sci. USA*, 1985, *82*, 968). Fluorescein has been linked to an oligonucleotide in the same manner as reported by Haralambidis, *et al.* (*Nucleic Acid Research*, 1987, *15*, 4857) and biotin in the same manner as described in PCT application PCT/US/02198. Fluorescein, biotin and pyrene were also linked in the same manner as reported by Telser *et al.* (*J. Am. Chem. Soc.*, 1989, *111*, 6966). A commercial reagent, Amino-Modifier-dT, from Glen Research Corporation (Sterling, VA) can be utilized to introduce pyrimidine nucleotides bearing similar linking groups into oligonucleotides.

Cholic acid linked to EDTA for use in radioscintigraphic imaging studies was reported by Betebenner *et al.* (*Bioconjugate Chem.*, 1991, *2*, 117); however, it is not known to link cholic acid to nucleosides, nucleotides or oligonucleotides. Despite these efforts and other research in the field, it is not known in the art to use lipophilic conjugation to alter the pharmacodynamic properties of an antisense compound, *i.e.*, an agent that works *via* a nucleotide sequence-dependent antisense mechanism.

OBJECTS OF THE INVENTION

It is one object of this invention to provide oligonucleotides and oligonucleosides functionalized to

include lipophilic moieties in order to produce lipophilic oligonucleotide and oligonucleoside conjugates which, relative to their unfunctionalized parent compounds, have improved biostability and altered biodistribution in mammals.

It is a further object of the invention to provide methods of modulating gene expression in cells, tissue(s) or organ(s) of a mammal using the lipophilic oligonucleotide and oligonucleoside conjugates of the invention.

It is a particular object of the invention to provide compositions for and methods of targeting antisense oligonucleotides to hepatic tissues and thereby preferentially modulating gene expression in the liver and associated tissues of a mammal.

BRIEF DESCRIPTION OF THE INVENTION

These and other objects are satisfied by the present invention, which provides oligonucleotides and oligonucleosides functionalized to include lipophilic moieties. In one aspect, the invention provides nucleosides having base portions and ribofuranosyl sugar portions. Such nucleosides bear at a 2'-O-position, a 3'-O-position, or a 5'-O-position a substituent having formula:

$$-R_A-N(R_{1a})(R_{1b})$$

where:

R_A is alkyl having from 1 to about 10 carbon atoms or R_A is $(CH_2-CH_2-Q-)_x$;

R_{1a} and R_{1b}, independently, are H, R_A, R_2, or an amine protecting group or have formula $C(X)-R_2$, $C(X)-R_A-R_2$, $C(X)-Q-R_A-R_2$, $C(X)-Q-R_2$;

R_2 includes a steroid molecule, a reporter molecule, a lipophilic molecule, a reporter enzyme, a peptide, a protein, or has formula $-Q-(CH_2CH_2-Q-)_x-R_3$;

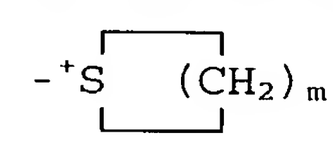
X is O or S;

each Q is, independently, is NH, O, or S;

x is 1 to about 200;

R_3 is H, R_A, C(O)OH, $C(O)OR_A$, $C(O)R_4$, R_A-N_3, R_A-NH_2, or R_A-SH; and

R_4 is Cl, Br, I, SO_2R_5 or has structure:



;

m is 2 to 7; and

R_5 is alkyl having 1 to about 10 carbon atoms.

In another aspect, the invention provides oligonucleotides and oligonucleosides comprising a plurality of linked nucleosides, wherein each nucleoside includes a ribofuranosyl sugar portion and a base portion and at least one (preferably more than one) of the nucleosides bears at a 2'-O-position, a 3'-O-position, or a 5'-O-position a substituent having formula $-R_A-N(R_{1a})(R_{1b})$.

In another aspect, the invention provides methods for preparing oligonucleotides and oligonucleosides comprising the steps of contacting nucleosides according to the invention for a time and under reaction conditions effective to form a covalent bond therebetween. In preferred embodiments, at least one of the nucleosides bears a phosphoramidate group at its 2'-O-position or at its 3'-O-position.

In other embodiments, compounds according to the invention are prepared by contacting a nucleoside, oligonucleotide or oligonucleoside with derivatizing reagents. For example, a nucleoside, oligonucleotide or

oligonucleoside bearing a 2'-hydroxy group, a 3'-hydroxy group, or a 5'-hydroxy group under basic conditions with a compound having formula L_1-R_A-$N(R_{1a})(R_{1b})$ wherein L_1 is a leaving group such as a halogen and at least one of R_{1a} and R_{1b} is an amine protecting group.

The present invention also provides methods for inhibiting the expression of particular genes in the cells of an organism, comprising administering to the organism a compound according to the invention. Also provided are methods for inhibiting transcription and/or replication of particular genes or for inducing degradation of particular regions of double stranded DNA in cells of an organism by administering to the organism a compound of the invention. Further provided are methods for killing cells or virus by contacting said cells or virus with a compound of the invention. The compound can be included in a composition that further includes an inert carrier for the compound.

In yet another aspect, the invention is directed to a method of modulating the expression of a nucleic acid in the hepatic system of a mammal, including the step of administering to the mammal an oligonucleotide, preferably an antisense oligonucleotide, which hybridizes to the nucleic acid to modulate the expression of the nucleic acid, wherein the oligonucleotide contains at least two sterol moieties covalently bound to the oligonucleotide. Preferably, at least one of the sterol moieties is a cholesteryl moiety. Most preferably, both of the sterol moieties are cholesteryl moieties. In addition, it preferable that the sterol moieties are bound at the 2'-O, 3'-O or 5'-O positions of the oligonucleotide.

In another aspect, the invention is directed to a method of preferentially targeting an oligonucleotide, preferably an antisense oligonucleotide, to liver cells, particularly endothelial cells, in a mammal, including the steps of covalently bonding to the oligonucleotide at least two sterol moieties to form a sterol-oligonucleotide conjugate; and administering the sterol-oligonucleotide conjugate to the mammal to preferentially target the oligonucleotide to the liver cells in the mammal to modulate the expression of a gene in the liver cells. Preferably, at least one of the sterol moieties is a cholesteryl moiety. Most preferably, both of the sterol moieties are cholesteryl moieties. In addition, it preferable that the sterol moieties are bound at the 2'-O, 3'-O or 5'-O positions of the oligonucleotide.

In yet another aspect, the invention is directed to a method of treating an animal having a hepatic disease or disorder associated with a protein encoded by a gene, including the step of administering to the mammal an oligonucleotide, preferably a antisense nucleotide, which hybridizes to the gene, wherein the oligonucleotide has at least two sterol moieties covalently bonded thereto. Preferably, at least one of the sterol moieties is a cholesteryl moiety. Most preferably, both of the sterol moieties are cholesteryl moieties. In addition, it preferable that the sterol moieties are bound at the 2'-O, 3'-O or 5'-O positions of the oligonucleotide.

In another aspect, the invention is directed to a composition, containing an oligonucleotide, preferably an antisense oligonucleotide, wherein the oligonucleotide has at least two sterol moieties covalently bonded thereto. Preferably, at least one of the sterol moieties is a cholesteryl moiety. Most preferably, both of the

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sterol moieties are cholesteryl moieties. In addition, it preferable that the sterol moieties are bound at the 2'-O, 3'-O or 5'-O positions of the oligonucleotide. Preferably, the oligonucleotide, in particular an antisense oligonucleotide, hybridizes to a gene encoding a protein that is overexpressed or abnormally expressed in hepatic tissues in the course of a disease or a disorder.

BRIEF DESCRIPTION OF THE DRAWINGS

The numerous objects and advantages of the present invention may be better understood by those skilled in the art by reference to the accompanying figures, in which:

Figure **1** shows the dose response for oligonucleotide inhibition of MDR1 message in transfected 3T3 cells. The MDR-3T3 cells were treated with 50 nM, 100 nM, 250 nM, 500 nM or 1000 nM concentrations of ISIS 5995 oligomer, or 1000 nM of ISIS 10221 scrambled control oligomer, for 24 hours in the presence of LIPOFECTIN^{R}. RNA was isolated, separated and probed with a 1.0 kB MDR1 cDNA fragment. The same blots were stripped and probed with a beta-actin probe to ensure uniform loading. Levels of MDR1 mRNA from the blots were quantitated using a PHOSPHORIMAGER^{TM} (Molecular Dynamics, Sunnyvale, CA) and the values are expressed as percent of control. The results represent mean values and standard errors of the mean from 5 independent experiments. Filled diamond, ISIS 5995; closed diamond, ISIS 10221.

Figure **2** shows the effect of cholesterol-conjugated phosphothioate oligonucleotides on P-glycoprotein surface expression. MDR 3T3 cells were treated with various

concentrations of oligonucleotides ISIS 11073 (5'-cholesterol 5995), or ISIS 12064 (5'-cholesterol 10221; scrambled control) for 48 hours in serum free medium. The cells were recovered and stained with an anti-P-glycoprotein monoclonal antibody directed against a surface epitope, followed by a phycoerythrin-conjugated second antibody. The level of cell surface fluorescence in viable cells was quantitated using a flow cytometer; light scatter parameters were set so as to exclude non-viable cells. A parallel experiment was done with cells treated with oligonucleotides ISIS 5995 and ISIS 10221 at 1 μm, in the presence of LIPOFECTIN^R. The data are presented as percent inhibition of P-glycoprotein expression, with the 100% level taken as that for untreated MDR 3T3 cells. The results represent means and standard errors for 6 determinations. Filled diamonds, ISIS 11073 (cholesterol-conjugated ISIS 5995); filled circles, ISIS 12064 (cholesterol-conjugated ISIS 10221; scrambled control); open diamonds, ISIS 5995; open triangles, ISIS 10221.

Figure **3** shows the results of experiments in which cells treated as described in Figure 2 were analyzed for Rhodamine 123 uptake. Subsequent to oligonucleotide treatment the cells were washed and then exposed to 1 ug/ml Rh 123 in serum free medium at 37°C. After 1 hour the cells were washed and the amount of Rh 123 accumulated by viable cells was quantitated using a flow cytometer. The results represent means and standard errors for 3 determinations. Symbols, as in Figure 2.

Figure **4** shows the uptake and intracellular distribution of effect of cholesterol-conjugated phosphothioate MDR1 oligonucleotides. MDR-3T3 cells were treated with 1 μm ISIS 13331 (5'-FITC, 3'-cholesterol

5995), or with 1 μm ISIS 13434 (5'-FITC 5995) for 2 hours in serum free medium at 37°C. Cells were harvested and the fluorescence profiles were determined using a flow cytometer; light scatter parameters were set so as to exclude non-viable cells. Solid line, free ISIS 13434 (5'-FITC 5995); dashed line, ISIS 13434 with Lipofectin^R; dotted line, ISIS 13331 (5'-FITC, 3'-cholesterol 5995).

Figure **5** shows the uptake and intracellular distribution of effect of cholesterol-conjugated phosphothioate MDR1 oligonucleotides of cells treated as in Figure 4 but for 18 hours instead of 2 hours. The 18 hour treatment with LIPOFECTIN^R resulted in some cells with very high levels (above 10^4 units) of fluorescence; these were accumulated in one channel and are shown as a vertical line at the right hand margin of the plot.

Figure **6** shows modulation of P-glycoprotein (PGP) expression by 2'-methoxyethoxy oligonucleotides as determined by flow cytometry essentially as described in Figure 2. Open boxes, ISIS 13753 (2'-methoxyethoxy derivative of ISIS 10221); closed circles, ISIS 13755 (2'-methoxyethoxy derivative of ISIS 5998); closed boxes, ISIS 13758 (2'-methoxyethoxy derivative of ISIS 5995).

Figure **7** is a graph showing mouse plasma concentrations of a control compound and two of the compounds of the invention. The plasma concentration is plotted versus time.

Figure **8** is a three-dimensional graph showing distribution of a control compound, ISIS 3082, among various tissues in the mouse. Specific tissues are shown on one axis, time on a second axis and percent of dose on the third axis. The compound was delivered by intravenous injection.

Figure 9 is a three-dimensional graph showing distribution of a compound of the invention, ISIS 9047, among various tissues in the mouse. Specific tissues are shown on one axis, time on a second axis and percent of dose on the third axis. The compound was delivered by intravenous injection.

Figure 10 is a three-dimensional graph showing distribution of a further compound of the invention, ISIS 8005, among various tissues in the mouse. Specific tissues are shown on one axis, time on a second axis and percent of dose on the third axis. The compound was delivered by intravenous injection.

Figure 11 shows data demonstrating the modulation of ICAM-1 mRNA levels in the livers of Balb/c mice by cholesterol-conjugated oligonucleotides. Terms: BASAL, untreated cells; LPS, lipopolysaccharide (ICAM-1 inducing agent); ISIS 3082, antisense phosphorothioate oligonucleotide targeted to mouse ICAM1 sequences; ISIS 8005, 5'-cholesterol conjugate of ISIS 3082; ISIS 13293, 5'-cholesterol-conjugated scrambled control oligonucleotide for ISIS 8005; the suffix "-30" indicates the dose (30 mg/kg) of oligonucleotide.

Figure 12 shows that the 5'-cholesterol ISIS 3082 analog (ISIS 8005) has no effect on c-raf and PECAM-1 mRNA levels in the murine liver. Terms: BASAL, untreated cells; lps, lipopolysaccharide (ICAM-1 inducing agent); ISIS 8005, 5'-cholesterol conjugated phosphorothioate antisense oligonucleotide targeted to mouse ICAM-1; ISIS 13293, 5'-cholesterol-conjugated scrambled control oligonucleotide for ISIS 8005; the suffix "-10" indicates the dose (10 mg/kg) of oligonucleotide.

Figure **13** shows that the 5'-cholesterol ISIS 3082 analog (ISIS 8005) has little effect on ICAM-1 levels in murine lungs. Terms: BASAL, untreated cells; LPS, lipopolysaccharide (ICAM-1 inducing agent); ISIS 3082, antisense phosphorothioate oligonucleotide targeted to mouse ICAM1 sequences; ISIS 8005, 5'-cholesterol conjugate of ISIS 3082; ISIS 13293, 5'-cholesterol-conjugated scrambled control oligonucleotide for ISIS 8005; the suffix "-30" indicates the dose (30 mg/kg) of oligonucleotide.

Figure **14** shows the histology of reversal of LPS-mediated ICAM-1 induction by cholesterol-conjugated antisense oligonucleotides.

Figure **15** shows that Balb/c mouse liver ICAM-1 mRNA levels can be modulated by a 3'-cholesterol-conjugated antisense oligonucleotide. Terms: basal, untreated cells; lps, lipopolysaccharide (ICAM-1 inducing agent); ISIS 9388, 3'-cholesterol conjugated phosphorothioate antisense oligonucleotide targeted to mouse ICAM-1; ISIS 13293, 5'-cholesterol-conjugated scrambled control oligonucleotide for ISIS 8005; ISIS 6777, control oligonucleotide targeted to VCAM-1; the suffixes "-1", "-10" and "-20" indicate the dose (1, 10 or 10 mg/kg, respectively) of oligonucleotide.

Figure **16** is a graph showing the plasma clearance of intravenously injected [^{3}H]ISIS-9389. Rats were intravenously injected with [^{3}H]ISIS-9389 at a dose of 1 mg/kg body weight. Blood samples were taken at the indicated times, and the radioactivity in the plasma was determined. Values are mean±S.E.M. of 3 rats.

Figure **17** is a graph showing a comparison of tissue uptake of intravenously injected [^{3}H]ISIS-3082, [^{3}H]ISIS-9388 and [^{3}H]ISIS-9389 (all at 1 mg/kg body weight). The

distribution of radioactivity over all tissues was determined at 90 min (ISIS-9389 and ISIS-3082) or 180 min (ISIS-9388) after injection. Radioactivity in the tissues is expressed as % of the radioactivity cleared from the circulation at the time of sampling, and constitutes the contribution of each tissue to the clearance. At the time of sampling, 97.9±0.3%, 88.9±2.3% and 94.4±0.6% of the injected dose of ISIS-3082, ISIS-9388 and ISIS-9389 had been cleared, respectively. Values are mean±S.E.M. of 3 rats.

Figure **18** is a graph showing the uptake of intravenously injected [^{3}H]ISIS-3082, [^{3}H]ISIS-9388 and [^{3}H]ISIS-9389 by liver cell types, all at a dose of 1 mg/kg body weight. One hour after injection, parenchymal, endothelial and Kupffer cells were isolated, and the amount of radioactivity associated with each cell type was determined. The contribution of each cell type to the total liver uptake was calculated from the uptake per mg of cell protein and the contribution of each cell type to the total liver protein. The percentage of the administered dose taken up by each cell type (when all oligonucleotide is cleared) was calculated from the contribution of each cell type to the total liver uptake and the contribution of the liver to the clearance (41.4±1.4%, 71.7±3.7% and 87.7±0.8% of the dose for ISIS-3082, ISIS-9388 and ISIS-9389, respectively). Values are mean±S.E.M. of 3-4 rats.

Figure **19** is a graph showing the effects of polyanions on the liver uptake of [^{3}H]ISIS-9389. Rats were intravenously injected with [^{3}H]ISIS-9389 at a dose of 1 mg/kg body weight. One minute prior to injection of the labeled ligand, the animals received 10 mg/kg polyinosinic acid (poly-I), 10 mg/kg polyadenylic acid

(poly-A), or an equal volume of saline solvent (2 ml/kg). At the indicated times, the amounts of radioactivity in the liver were determined. Values are mean±S.E.M. of 3-4 rats.

Figure **20** is a graph showing the association of ISIS-9389, ISIS-9388 and ISIS-3082 with plasma components. [^{3}H]-labeled oligonucleotides were incubated at 37°C with rat plasma at a concentration of 20 μg/ml. After 30 min, aliquots of the incubation mixtures were subjected to size exclusion chromatography. Fractions were collected and assayed for radioactivity. The results are expressed as percentages of the recovered amounts (recoveries>95%). The elution volumes of LDL, HDL, serum albumin and free oligodeoxynucleotide(ODN)are indicated by arrows.

Figure **21A-21B** are graphs showing the association of ISIS-9389 with LDL and HDL. [^{3}H]ISIS-9389 (20 μg/ml) was incubated at 37°C with 0.2 mg/ml rat ^{125}I-HDL (Fig. **21B**). After 30 min, aliquots of the incubation mixture were subjected to size exclusion chromatography. Fractions were assayed for ^{3}H (•) and ^{125}I (O). The results are expressed as percentages of the recovered radioactivity (recoveries >80%). The elution volumes of LDL, HDL and free ODN are indicated by arrows.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides nucleosides, oligonucleotides and oligonucleosides functionalized to include lipophilic moieties in order to produce lipophilic oligonucleotide and oligonucleoside conjugates which, relative to their unfunctionalized parent compounds, have improved biostability and altered

biodistribution in mammals. The nucleoside subunits can be "natural" or "synthetic" moieties. Each nucleoside is formed from a naturally occurring or synthetic base and a naturally occurring or synthetic pentofuranosyl sugar group.

The term "oligonucleotide" refers to a polynucleotide formed from a plurality of linked nucleotide units. The nucleotide units each include a nucleoside unit. In the context of this invention, the term "oligonucleoside" refers to a plurality of nucleoside units that are linked together. In a generic sense, since each nucleotide unit of an oligonucleotide includes a nucleoside therein, the term "oligonucleoside" can be considered to be inclusive of oligonucleotides (*i.e.*, nucleosides linked together via phosphate linking groups). In a further sense, the term "oligonucleoside" also refers to a plurality of nucleosides that are linked together via linkages other than phosphate linkages. The term "oligonucleoside" thus effectively includes naturally occurring species or synthetic species formed from naturally occurring subunits. For brevity, the term "oligonucleoside" will be used as encompassing both phosphate linked (oligonucleotides) and non-phosphate linked polynucleoside species.

Oligonucleosides according to the invention also can include modified subunits. Representative modifications include modification of a heterocyclic base portion of a nucleoside or a sugar portion of a nucleoside. Exemplary modifications are disclosed in the following United States Patent Applications: Serial No. 07/835,932, filed March 5, 1992, now U.S. Patent No. 5,670,633, entitled Sugar Modified Oligonucleotides That Detect And Modulate Gene Expression; Serial No. 558,663, filed July 27, 1990,

now U.S. Patent No. 5,138,045, entitled Novel Polyamine Conjugated Oligonucleotides; Serial No. 558,806, filed July 27, 1991, entitled Nuclease Resistant Pyrimidine Modified Oligonucleotides that Detect and Modulate Gene Expression and Serial No. PCT/US91/00243, filed January 11, 1991, entitled Compositions and Methods for Detecting and Modulating RNA Activity. Teachings regarding the synthesis of particular modified oligonucleotides may also be found in the following U.S. patents or pending patent applications, each of which is commonly assigned with this application: U.S. Patents Nos. 5,138,045 and 5,218,105, drawn to polyamine conjugated oligonucleotides; U.S. Patent No. 5,212,295, drawn to monomers for the preparation of oligonucleotides having chiral phosphorus linkages; U.S. Patents Nos. 5,378,825 and 5,541,307, drawn to oligonucleotides having modified backbones; U.S. Patent No. 5,386,023, drawn to backbone modified oligonucleotides and the preparation thereof through reductive coupling; U.S. Patent No. 5,457,191, drawn to modified nucleobases based on the 3-deazapurine ring system and methods of synthesis thereof; U.S. Patent No. 5,459,255, drawn to modified nucleobases based on N-2 substituted purines; U.S. Patent No. 5,521,302, drawn to processes for preparing oligonucleotides having chiral phosphorus linkages; U.S. Patent No. 5,539,082, drawn to peptide nucleic acids; U.S. Patent No. 5,554,746, drawn to oligonucleotides having β-lactam backbones; U.S. Patent No. 5,571,902, drawn to methods and materials for the synthesis of oligonucleotides; U.S. Patent No. 5,578,718, drawn to nucleosides having alkylthio groups, wherein such groups may be used as linkers to other moieties attached at any of a variety of positions of the nucleoside; U.S. Patents Nos. 5,587,361 and 5,599,797,

drawn to oligonucleotides having phosphorothioate linkages of high chiral purity; U.S. Patent No. 5,506,351, drawn to processes for the preparation of 2'-O-alkyl guanosine and related compounds, including 2,6-diaminopurine compounds; U.S. Patent No. 5,587,469, drawn to oligonucleotides having N-2 substituted purines; U.S. Patent No. 5,587,470, drawn to oligonucleotides having 3-deazapurines; U.S. Patents Nos. 5,223,168, issued June 29, 1993, and 5,608,046, both drawn to conjugated 4'-desmethyl nucleoside analogs; U.S. Patent Nos. 5,602,240, and 5,610,289, drawn to backbone modified oligonucleotide analogs; and U.S. patent application Serial No. 08/383,666, filed February 3, 1995, and U.S. Patent No. 5,459,255, drawn to, *inter alia*, methods of synthesizing 2'-fluoro-oligonucleotides.

The term oligonucleoside thus refers to structures that include modified portions, be they modified sugar moieties or modified base moietieS that function similarly to natural bases and natural sugars. Representative modified bases include deaza or aza purines and pyrimidines used in place of natural purine and pyrimidine bases; pyrimidines having substituent groups at the 5 or 6 position; and purines having altered or replacement substituent groups at the 2, 6 or 8 positions. Representative modified sugars include carbocyclic or acyclic sugars, sugars having substituent groups at their 2' position, and sugars having substituents in place of one or more hydrogen atoms of the sugar. Other altered base moieties and altered sugar moieties are disclosed in United States Patent 3,687,808 and PCT application PCT/US89/02323.

Altered base moieties or altered sugar moieties also include other modifications consistent with the spirit of

this invention. Such oligonucleosides are best described as being structurally distinguishable from yet functionally interchangeable with naturally occurring or synthetic wild type oligonucleotides. All such oligonucleosides are comprehended by this invention so long as they function effectively to mimic the structure of a desired RNA or DNA strand.

For use in antisense methodology, the oligonucleosides of the invention preferably comprise from about 10 to about 30 subunits. It is more preferred that such oligonucleosides comprise from about 15 to about 25 subunits. As will be appreciated, a subunit is a base and sugar combination suitably bound to adjacent subunits through, for example, a phosphorus-containing (*e.g.*, phosphodiester) linkage or some other linking moiety. The nucleosides need not be linked in any particular manner, so long as they are covalently bound. Exemplary linkages are those between the 3'- and 5'-positions or 2'- and 5'-positions of adjacent nucleosides. Exemplary linking moieties are disclosed in the following references: Beaucage, *et al.*, *Tetrahedron*, 1992, *48*, 2223 and references cited therein; and United States Patents and applications: serial No. 07/703,619, filed May 21, 1991 (now U.S. Patent No. 5,378,825); Serial No. 07/903,160, filed June 24, 1992 (now U.S. Patent No. 5,623,070); serial No. 039,979, filed March 20, 1993 (currently pending as continuation application serial No. 08/317,289, filed October 3, 1994); Serial No. 08/039,846, filed March 30, 1993 and serial No. 08/392,675 filed February 23, 1995, (now U.S. Patent No. 5,677,437); and serial No. 08/040,933, filed March 31, 1993 (now U.S. Patent No. 5,618,704). Each of the foregoing Patents or applications is assigned to the

assignee of this invention, and the disclosure of each is incorporated herein by reference.

It is preferred that the RNA or DNA portion which is to be modulated using oligonucleosides of the invention be preselected to comprise that portion of DNA or RNA which codes for the protein whose formation or activity is to be modulated. The targeting portion of the composition to be employed is, thus, selected to be complementary to the preselected portion of DNA or RNA, that is, to be an antisense oligonucleoside for that portion.

In accordance with one preferred embodiment of this invention, the compounds of the invention hybridize to mRNA encoding ICAM-1 (intercellular adhesion molecule 1). ICAM-1 is a cell surface glycoprotein expressed primarily in endothelial cells that binds other cells, such as neutrophils, expressing cell surface antigens such as LFA-1 (Kishimoto *et al.*, *Adv. Immunol.*, 1989, *46*, 149). Several lines of experimentation indicate that ICAM-1 plays an important role during various inflammatory responses (Bochner *et al.*, *J. Exp. Med.*, 1991, *173*, 1553; Carlos *et al.*, *Blood*, 1991, *77*, 2266). By modulating such responses according to the compositions and methods of the present invention, undesirable inflammatory responses are mediated. In another preferred embodiment of the invention, the compounds of the invention hybridize to MDR1 (multidrug resistance) mRNA encoding a membrane protein (P-glycoprotein) that functions as an ATP driven efflux pump. Hyperproliferative cells may become resistant to anticancer agents due to an overabundance of one or more nucleic acids (*i.e.*, mRNA or DNA) encoding one or more such MDR proteins. By modulating this resistance

according to the compositions and methods of the present invention, resistant cells are resensitized to such anticancer agents. Accordingly, the compositions and methods of the invention act to enhance the treatment of abnormal cell proliferation and tumor formation with anticancer agents. In further embodiments of the invention, the compounds of the invention hybridize to HIV mRNA encoding the tat protein, or to the TAR region of HIV mRNA. Other preferred compounds are complementary to sequences for herpes, papilloma and other viruses, or to sequences corresponding to cellular oncogenes, mediators of the immune response of an animal and other host-encoded functions.

The nucleosides and oligonucleosides of the invention can be used in diagnostics, therapeutics and as research reagents and kits. They can be used in pharmaceutical compositions by including a suitable pharmaceutically acceptable diluent or carrier. They further can be used for treating organisms having a disease characterized by the undesired production of a protein. The organism should be contacted with an oligonucleotide having a sequence that is capable of specifically hybridizing with a strand of nucleic acid coding for the undesirable protein. Treatments of this type can be practiced on a variety of organisms ranging from unicellular prokaryotic and eukaryotic organisms to multicellular eukaryotic organisms. Any organism that utilizes DNA-RNA transcription or RNA-protein translation as a fundamental part of its hereditary, metabolic or cellular control is susceptible to therapeutic and/or prophylactic treatment in accordance with the invention. Seemingly diverse organisms such as bacteria, yeast, protozoa, algae, all plants and all higher animal forms,

including warm-blooded animals, can be treated. Further, each cell of multicellular eukaryotes can be treated since they include both DNA-RNA transcription and RNA-protein translation as integral parts of their cellular activity. Many of the organelles (*e.g.*, mitochondria and chloroplasts) of eukaryotic cells also include transcription and translation mechanisms. Thus, single cells, cellular populations or organelles can also be included within the definition of organisms that can be treated with therapeutic or diagnostic oligonucleotides.

As used herein, therapeutics is meant to include the eradication of a disease state, by killing an organism or by control of erratic or harmful cellular growth or expression. The invention is also drawn to the administration of biologically active oligonucleotides having biological activity to cultured cells, isolated tissues and organs and animals. By being "biologically active," it is meant that the oligonucleotide functions to modulate the expression of one or more genes in cultured cells, isolated tissues or organs and/or animals. Such modulation can be achieved by an antisense oligonucleotide by a variety of mechanisms known in the art, including but not limited to transcriptional arrest; effects on RNA processing (capping, polyadenylation and splicing) and transportation; enhancement of cellular degradation of the target nucleic acid; and translational arrest (Crooke *et al.*, *Exp. Opin. Ther. Patents*, 1996, *6*:855).

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. In general, for therapeutics, a patient in need of such therapy is administered an oligonucleotide in accordance with the

invention, commonly in a pharmaceutically acceptable carrier, in doses ranging from 0.01 μg to 100 g per kg of body weight depending on the age of the patient and the severity of the disorder or disease state being treated. Further, the treatment regimen may last for a period of time that will vary depending upon the nature of the particular disease or disorder, its severity and the overall condition of the patient, and may extend from once daily to once every 20 years. Following treatment, the patient is monitored for changes in his/her condition and for alleviation of the symptoms of the disorder or disease state. The dosage of the oligonucleotide may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disorder or disease state is observed, or if the disorder or disease state has been ablated.

In some cases it may be more effective to treat a patient with an oligonucleotide of the invention in conjunction with other traditional therapeutic modalities in order to increase the efficacy of a treatment regimen. In the context of the invention, the term "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight, once or more daily, to once every 20 years.

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is

desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, or intrathecal or intraventricular administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions that may also contain buffers, diluents and other suitable additives.

Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be

effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 μg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years.

In one aspect, the present invention is directed to nucleosides and oligonucleosides that bear at least one amine-containing substituent at a position. Such substituents preferably have formula $-R_A-N(R_{1a})(R_{1b})$ and are appended at 2'-O-, 3'-O-, and/or 5'-O-positions

Each R_A according to the invention is an alkyl moiety independently selected to have 1 to about 10 carbon atoms or R_A is a polyether, a polythioether or polyalkylamine. The term "alkyl" is intended to include straight chain and branched hydrocarbons. The preferred length of these hydrocarbons is 1 to about 7 carbon atoms.

R_{1a} and R_{1b} according to the invention are H, R_A, R_2, an amine protecting group, or have formula $C(X)-R_2$, $C(X)-R_A-R_2$, $C(X)-Q-R_A-R_2$, $C(X)-Q-R_2$. Protecting groups are known *per se* as chemical functional groups that can be selectively appended to and removed from functionalities, such as amine groups. These groups are present in a chemical compound to render such functionality inert to chemical reaction conditions to which the compound is exposed. *See, e.g.*, Greene and Wuts, Protective Groups in Organic Synthesis, 2d edition, John Wiley & Sons, New York, 1991. Numerous amine protecting groups are known in the art, including, but not limited to: phthalimide (PHTH), trifluoroacetate (triflate), allyloxycarbonyl (Alloc), benzyloxycarbonyl (CBz), chlorobenzyloxycarbonyl, t-butyloxycarbonyl (Boc), fluorenylmethoxycarbonyl (Fmoc), and isonicotinyloxycarbonyl (i-Noc) groups. (*see, e.g.*, Veber and Hirschmann, *et al.*, *J. Org. Chem.*, 1977, *42*, 3286 and

Atherton, *et al.*, The Peptides, Gross and Meienhofer, Eds, Academic Press; New York, 1983; Vol. 9 pp. 1- 38).

R_2 can include a steroid molecule, a reporter molecule, a lipophilic molecule, a reporter enzyme, a peptide, a protein (*i.e.*, a substituent consisting essentially of same), or a molecule having formula -Q-$(CH_2CH_2$-Q-$)_x$-R_3. For the purposes of this invention the terms "reporter molecule" and "reporter enzyme" are inclusive of those molecules or enzymes that have physical or chemical properties that allow them to be identified in gels, fluids, whole cellular systems, broken cellular systems and the like utilizing physical properties such as spectroscopy, radioactivity, colorimetric assays, fluorescence, and specific binding. Steroids include those chemical compounds that contain a perhydro-1,2-cyclopentanophenanthrene ring system. Proteins and peptides are utilized in their usual sense as polymers of amino acids.

Normally peptides comprise such polymers that contain a smaller number of amino acids per unit molecule than do the proteins. Lipophilic molecules include naturally-occurring and synthetic aromatic and non-aromatic moieties such as fatty acids, esters, alcohols and other lipid molecules, substituted aromatic groups such as dinitrophenyl groups, cage structures such as adamantane and buckminsterfullerenes, and aromatic hydrocarbons such as benzene, perylene, phenanthrene, anthracene, naphthalene, pyrene, chrysene, and naphthacene.

Particularly useful as steroid molecules are the bile acids including cholic acid, deoxycholic acid and dehydrocholic acid; steroids including cortisone, digoxigenin, testosterone and cholesterol and even

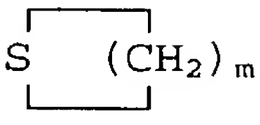
cationic steroids such as cortisone having a trimethylaminomethyl hydrazide group attached via a double bond at the 3 position of the cortisone rings.

Particularly useful as reporter molecules are biotin, dinitrophenyl, and fluorescein dyes. Particularly useful as lipophilic molecules are alicyclic hydrocarbons, saturated and unsaturated fatty acids, waxes, terpenes and polyalicyclic hydrocarbons including adamantane and buckminsterfullerenes. Particularly useful as reporter enzymes are alkaline phosphatase and horseradish peroxidase. Particularly useful as peptides and proteins are sequence-specific peptides and proteins including phosphodiesterase, peroxidase, phosphatase and nuclease proteins. Such peptides and proteins include SV40 peptide, RNaseA, RNase H and Staphylococcal nuclease. Particularly useful as terpenoids are vitamin A, retinoic acid, retinal and dehydroretinol.

Some preferred positions of attachment of the lipophilic molecules, particularly steroids, include the 3′ position of the sugar of the 3′ terminal nucleotide, the 5′ position of the sugar of the 5′ terminal nucleotide and the 2′ position of the sugar of any nucleotide. The N6 position of a purine nucleotide may also be used to link a steroid to the nucleotide.

R_2 also can have formula $-Q-(CH_2CH_2-Q-)_x-R_3$, where Q is O, S, or NH. Subscript x can be 1 to about 200, preferably about 20 to about 150, more preferably about 10 to about 50. Preferably, Q are selected to be O, such that R_2 constitutes a poly(ethyleneglycol) (PEG) group (*i.e.*, R_3 = H) or a functionalized derivative thereof (*e.g.*, R_3 = C(O)Cl). R_3 can be H, R_A, C(O)OH, $C(O)OR_A$, $C(O)R_4$, R_A-N_3, R_A-NH_2 or R_A-SH where R_4 is F, Cl, Br, I,

SO_2R_5 or a small thio-containing heterocycle having structure:



where m is 2 to 7. Representative PEG-containing R_2 groups are disclosed by Ouchi, *et al.*, *Drug Design and Discovery*, 1992, *9*, 93, Ravasio, *et al.*, *J. Org. Chem.*, 1991, *56*, 4329, and Delgardo *et. al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1992, *9*, 249.

Oligonucleosides according to the invention can be assembled in solution or through solid-phase reactions, for example, on a suitable DNA synthesizer utilizing nucleosides according to the invention and/or standard nucleotide precursors. The nucleosides and nucleotide precursors can already bear alkylamino groups or can be later modified to bear such groups.

In the former case, compounds according to the invention are prepared by, for example, reacting nucleosides bearing at least one free 2'-, 3'-, or 5'-hydroxyl group under basic conditions with a compound having formula L_1-$(CH_2)_n$-$N(R_{1a})(R_{1b})$ where L_1 is a leaving group and at least one of R_{1a} and R_{1b} is an amine protecting group. Displacement of the leaving group through nucleophilic attack of an oxygen anion produces the desired amine derivative. Leaving groups according to the invention include but are not limited to halogen, alkylsulfonyl, substituted alkylsulfonyl, arylsulfonyl, substituted arylsulfonyl, hetercyclcosulfonyl or trichloroacetimidate. A more preferred group includes chloro, fluoro, bromo, iodo, p-(2,4-dinitroanilino)-benzenesulfonyl, benzenesulfonyl, methylsulfonyl (mesylate), p-methylbenzenesulfonyl (tosylate), p-bromobenzenesulfonyl, trifluoromethylsulfonyl (triflate),

trichloroacetimidate, acyloxy, 2,2,2-trifluoroethanesulfonyl, imidazolesulfonyl, and 2,4,6-trichlorophenyl, with bromo being preferred.

Suitably protected nucleosides can be assembled into an oligonucleosides according to known techniques. *See, e.g.*, Beaucage, *et al.*, *Tetrahedron*, 1992, *48*, 2223.

Oligonucleosides according to the invention also can be prepared by assembling an oligonucleoside and appending alkylamino functionality thereto. For example, oligonucleosides having free hydroxyl groups can be assembled according to known techniques and then reacted with a reagent having formula L_1-$(CH_2)_n$-$N(R_{1a})(R_{1b})$. As will be recognized, however, greater selectivity can be achieved in terms of placement of alkylamino functionality within an oligonucleoside by introducing such functionality, as discussed above, on selected nucleosides and then using both the selected nucleosides and other nucleosides to construct an oligonucleoside.

Once assembled, an oligonucleoside bearing one or more groups having formula -R_A-$N(R_{1a})(R_{1b})$ wherein at least one of R_{1a} and R_{1b} is a protecting group is treated with reagents effective to remove the protecting group. Once deprotected, the oligonucleoside can be contacted with electrophilic moieties such as, for example, succinimidyl esters and other activated carboxylic acids including C(=O)-O-succinimide and C(=O)-O-pentafluorophenyl, isothiocyanates, sulfonyl chlorides, halacetamides, phospholipid carbocyclic acid active esters, o-phenanthroline-5-iodoacetamide, fluorescein isothiocyanate, 1-pyrene butyric acid-N-hydroxy succinimide ester and carboxylic acid derivatives of PNA (carboxylic acid derivatives of peptide nucleic acids). Preferred electrophilic moieties include cholesteryl-3-

hemisuccinate-N-hydroxy succinimide ester, pyrene-1-butyric acid-N-hydroxy succinimide ester and polyethylene glycol-propionic acid-N-hydroxy succimide ester.

Thus, the invention first builds the desired linked nucleoside sequence in the normal manner on the DNA synthesizer. One or more (preferably two or more) of the linked nucleosides are then functionalized or derivatized with the lipophilic steroid, reporter molecule, lipophilic molecule, reporter enzyme, peptide or protein.

The following Examples illustrate the invention and are not intended to limit the same. Those skilled in the art will recognize, or be able to ascertain through routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of the present invention. For example, those skilled in the art will appreciate that it is also possible to synthesize modified oligonucleotides appropriate for use in the methods of the invention, such as, for example, cholesterol-conjugated oligonucleotides, by using modified amidites that have become available subsequent to the earliest priority date of the present application to prepare commercially available modified amidites and controlled-pore glass (CPG) products such as those available from Glen Research (Sterling, VA; *see*, for example, Agrawal, Chapter 3 *In: Methods in Molecular Biology, Vol. 26: Protocols for Oligonucelotide Conjugates*, Agrawal, ed., Humana Press Inc., Totowa, NJ (1994) pages 93-120.

EXAMPLES

EXAMPLE 1: PREPARATION OF OLIGONUCLEOTIDES HAVING 2'-PROTECTED-AMINE TERMINATING LINKING GROUP

[redacted]

A. Preparation of 5'-Dimethoxytrityl-2'-(O-Pentyl-N-phthalimido)-2'-Deoxyadenosine Phosphoramidite

To introduce a functionalization at the 2' position of nucleotides within desired oligonucleotide sequences, 5'-dimethoxytrityl-2'-(O-pentyl-N-phthalimido)-2'-deoxy-adenosine phosphoramidite was utilized to provide a linking group attached to the 2' position of nucleotide components of an oligonucleotide. This compound was synthesized generally in accordance with the procedures of PCT Application WO 91US00243 and U.S. Patent No. 6,262,241 starting from adenosine. Briefly, this procedure treats adenosine with NaH in dimethylformamide (DMF) followed by treatment with N-(5-bromopentyl)phthalimide. Further treatment with $(CH_3)_3SiCl$, Ph-C(O)-Cl and NH_4OH yields N6-benzyl protected 2'-pentyl-N-phthalimido functionalized adenosine. Treatment with DIPA and CH_2Cl_2 adds a DMT blocking group at the 5' position. Finally phosphitylation gives the desired phosphoramidite compound. This compound was utilized in the DNA synthesizer as a 0.09M solution in anhydrous CH_3CN. Oligonucleotide synthesis was carried out in either an ABI 390B or an ABI 394 synthesizer employing the standard synthesis cycles with an extended coupling time of 10 minutes during coupling of Compound 2 into the oligonucleotide sequence. Coupling efficiency of greater than 98% was observed.

B. 2'-Protected-Amine Linking Group Containing Phosphodiester Oligonucleotides

The following oligonucleotides having phosphodiester inter-nucleotide linkages were synthesized (throughout the disclosure, unless otherwise indicated, all

oligonucleotide sequences are listed in a standard 5' to 3' order from left to right):

Oligomer 9: 5'-TA*G-3';
Oligomer 10: 5'-CCA*G-3';
Oligomer 11: 5'-GGC-TGA*-CTG-CG-3' (SEQ ID NO:1);
Oligomer 12: 5'-CTG-TCT-CCA*-TCC-TCT-TCA-CT (SEQ ID NO:2);
Oligomer 13: 5'-CTG-TCT-CCA*-TCC-TCT-TCA*-CT (SEQ ID NO:26);

wherein A* represents a nucleotide functionalized to incorporate a pentyl-N-phthalimido functionality. Oligomers 12 and 13 are antisense compounds to the E2 region of the bovine papilloma virus-1 (BPV-1). Oligomers 12 and 13 have the same sequence as Oligomer 3 in U.S. Patent 6,265,558, except for the 2' modification. The oligonucleotides were synthesized in either a 10 μmol scale or a 3 x 1 μmol scale in the "Trityl-On" mode. Standard deprotection conditions (30% NH_4OH, 55°C, 24 hours) were employed. The oligonucleotides were purified by reverse phase HPLC (Waters Delta-Pak C_4 15 μm, 300A, 25x100 mm column equipped with a guard column of the same material). They were detritylated and further purified by size exclusion using a Sephadex G-25 column. NMR analyses by both proton and phosphorus NMR confirmed the expected structure for the Oligomers 9 and 10.

C. 2'-Protected-Amine Linking Group Containing Phosphorothioate Oligonucleotides

The following oligonucleotides having phosphorothioate inter-nucleotide linkages were synthesized:

Oligomer 14: $T_sT_sG_s$-$C_sT_sT_s$-$C_sC_sA^*_s$-$T_sC_sT_s$-$T_sC_sC_s$-$T_sC_sG_s$-T_sC(SEQ ID NO:3);

Oligomer 15: $T_sG_sG_s$-$G_sA_sG_s$-$C_sC_sA_s$-$T_sA_sG_s$-$C_sG_sA^*_s$-G_sG_sC(SEQ ID NO:4); and

Oligomer 16: $T_sG_sG_s$-$G_sA^*_sG_s$-$C_sC_sA^*_s$-$T_sA^*_sG_s$-$C_sG_sA^*_s$-G_sG_sC(SEQ ID NO:66);

wherein A* represents a nucleotide functionalized to incorporate a pentyl-N-phthalimido functionality and the subscript "s" represents a phosphorothioate inter-nucleotide backbone linkage. Oligomer 14 is an antisense compound directed to the E2 region of the bovine papilloma virus-1 (BPV-1). Oligomers 15 and 16 are antisense compounds to ICAM. Oligomer 14 has the same sequence as Oligomer 3 in U.S. Patent 6,265,558, except for the 2' modification whereas Oligomers 15 and 16 have the same sequence as Oligomer 4 in U.S. Patent 6,265,558 except for the 2' modification. These oligonucleotides were synthesized as per the method of Example 1(B) except during the synthesis, for oxidation of the phosphite moieties, the Beaucage reagent (*i.e.*, 3H-1,2-benzodithioate-3-one 1,1-dioxide; Radhakrishnan *et al.*, *J. Am. Chem. Soc.*, 1990, *112*, 1253) was used as a 0.24 M solution in anhydrous CH_3CN solvent. The oligonucleotides were synthesized in the "Trityl-On" mode and purified by reverse phase HPLC utilizing the purification procedure of Example 1(B).

D. 2'-O-Methyl Derivatized, 2'-Protected-Amine Linking Group Containing RNA Oligonucleotides

The following oligonucleotides having 2'-O-methyl groups on each nucleotide not functionalized with a 2'-protected amine functionalization were synthesized:

[redacted]

Oligomer 17: 5'-CCA-A*GC-CUC-AGA (SEQ ID NO:24); and
Oligomer 18: 5'-CCA-GGC-UCA-GA*T (SEQ ID NO:25);

wherein A* represents a nucleotide functionalized to incorporate a pentyl-N-phthalimido functionality and where the remaining nucleotides except the 3'-terminus nucleotide are each 2'-O-methyl derivatized nucleotides. The 3'-terminus nucleotide in both Oligomers 17 and 18 is a 2'-deoxy nucleotide. Both Oligomers 17 and 18 are antisense compounds to the HIV-1 TAR region. The oligonucleotides were synthesized as per the method of Example 6 in U.S. Patent 6,265,558 (utilizing Compound 2 thereof) for introduction of the nucleotides containing the pentyl-N-phthalimido functionality and appropriate 2-O-methyl phosphoramidite nucleotides from Chemgenes Inc. (Needham, MA) for the remaining RNA nucleotides. The 3'-terminus terminal 2'-deoxy nucleotides were standard phosphoamidites utilized for the DNA synthesizer. The oligonucleotides were deprotected and purified as per the method of Example 1(B).

EXAMPLE 2: FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE 2' POSITION

A. Functionalization with Biotin

1. Single Site Modification

About 10 O.D. units (A_{260}) of Oligomer 12 (approximately 60 nmols based on the calculated extinction coefficient of 1.6756×10^5) were dried in a microfuge tube. The oligonucleotide was dissolved in 200 μl of 0.2 M $NaHCO_3$ buffer and D-biotin-N-hydroxysuccinimide ester (2.5 mg, 7.3 μmols) (Sigma, St.

Louis, MO) was added followed by 40 μl DMF. The solution was let stand overnight. The solution was applied to a Sephadex G-25 column (0.7 x 15 cm) and the oligonucleotide fractions were combined. Analytical HPLC showed nearly 85% conversion to the product. The product was purified by HPLC (Waters 600E with 991 detector, Hamilton PRP-1 column 0.7 x 15 cm; solvent A: 50 mM TEAA pH 7.0; B: 45 mM TEAA with 80% acetonitrile: 1.5 ml flow rate: Gradient: 5% B for first 5 minutes, linear (1%) increase in B every minute thereafter) and further desalted on Sephadex G-25 to give the oligonucleotide:

Oligomer 19: 5'-CTG-TCT-CCA*-TCC-TCT-TCA-CT (SEQ ID NO:2);

wherein A* represents a nucleotide functionalized to incorporate a biotin functionality linked via a 2'-O-pentyl-amino linking group to the 2' position of the designated nucleotide. HPLC retention times are shown in Table 1 below.

2. Multiple Site Modification

About 10 O.D. units (A_{260}) of Oligomer 13 (approximately 60 nmols) was treated utilizing the method of Example 8(A)(1) in U.S. Patent 6,265,558 with D-biotin-N-hydroxysuccinimide ester (5 mg) in 300 μl of 0.2 M $NaHCO_3$ buffer/ 50 μl DMF. Analytical HPLC showed 65% of double-labeled oligonucleotide product and 30% of single labeled products (from the two available reactive sites). HPLC and Sephadex G-25 purification gave the oligonucleotide:

Oligomer 20: 5'-CTG-TCT-CCA*-TCC-TCT-TCA*-CT (SEQ ID NO:65);

wherein A* represents nucleotides functionalized to incorporate a biotin functionality linked via a 2'-O-pentyl-amino linking group to the 2' position of the designated nucleotide. HPLC retention times for this product (and its accompanying singly labeled products) are shown in Table 1 below.

B. Functionalization with Fluorescein

1. Single Site Modification

A 1M Na_2CO_3/1M $NaHCO_3$ buffer (pH 9.0) was prepared by adding 1M $NaHCO_3$ to 1 M Na_2CO_3. A 200 μl portion of this buffer was added to 10 O.D. units of Oligomer 12 in a microfuge tube. A 10 mg portion of fluorescein-iso-cyanate in 500 μl DMF was added to give a 0.05 M solution. A 100 μl portion of the fluorescein solution was added to the oligonucleotide solution in the microfuge tube. The tube was covered with aluminum foil and let stand overnight. The reaction mixture was applied to a Sephadex G-25 column (0.7 x 20 cm) that had been equilibrated with 25% (v/v) ethyl alcohol in water. The column was eluted with the same solvent. Product migration could be seen as a yellow band well separated from dark yellow band of the excess fluorescein reagent. The fractions showing absorption at 260 nm and 485 nm were combined and purified by HPLC as per the purification procedure of Example 2(A)(1). Analytical HPLC indicated 81% of the desired doubly functionalized oligonucleotide. The product was lyophilized and desalted on Sephadex to give the oligonucleotide:

Oligomer 21: 5'-CTG-TCT-CCA*-TCC-TCT-TCA-CT (SEQ ID NO:74);

wherein A* represents a nucleotide functionalized to incorporate a fluorescein functionality linked via a 2'-O-pentyl-amino linking group to the 2' position of the designated nucleotide. HPLC retention times are shown in Table 1 below.

2. Multiple Site Modification

A 10 O.D. unit (A_{260}) portion of Oligomer 13 (from Example 1) was dissolved in 300 μl of the 1M Na_2HCO_3/ 1M Na_2CO_2 buffer of Example 2(B)(1) and 200 μl of the fluorescein-isothiocyanate stock solution of Example 2(B)(1) was added. The resulting solution was treated as per Example 2(B)(1). Analytical HPLC indicated 61% of doubly labeled product and 38% of singly labeled products. Work up of the reaction gave the oligonucleotide:

Oligomer 22: 5'-CTG-TCT-CCA*-TCC-TCT-TCA*-CT (SEQ ID NO:75);

wherein A* represents nucleotides functionalized to incorporate a fluorescein functionality linked via a 2'-O-pentyl-amino linking group to the 2' position of the designated nucleotide. HPLC retention times are shown in Table 1 below.

C. Functionalization with Cholic Acid

1. Single Site Modification

A 10 O.D. unit (A_{260}) portion of Oligomer 12 was treated with cholic acid-NHS ester (Compound 1 in U.S. Patent 6,265,558, 5 mg, 9.9 μmols) in 200 μl of 0.2 M $NaHCO_3$ buffer/40 μl DMF. The reaction mixture was heated for 16 hours at 45°C. The product was isolated as per the method of Example 2(B)(1). Analytical HPLC indicated greater than 85% product formation. Work up of the reaction gave the oligonucleotide:

Oligomer 23: 5'-CTG-TCT-CCA*-TCC-TCT-TCA-CT (SEQ ID NO:76);

wherein A* represents a nucleotide functionalized to incorporate a cholic acid functionality linked via a 2'-O-pentyl-amino linking group to the 2' position of the designated nucleotide. HPLC retention times are shown in Table 1 below.

2. Multiple Site Modification

A 10 O.D. unit (A_{260}) portion of Oligomer 13 (*see*, Example 1) was treated with cholic acid-NHS ester (Compound 1 in U.S. Patent 6,265,558, 10 mg, 19.8 μmols) in 300 μl of 0.2 M $NaHCO_3$ buffer/ 50 μl DMF. The reaction mixture was heated for 16 hours at 45°C. The product was isolated as per the method of Example 2(A)(1). Analytical HPLC revealed 58% doubly labeled product, 17% of a first singly labeled product and 24% of a second singly labeled product. Work up as per Example 2(A)(1) gave the oligonucleotide:

Oligomer 24: 5'-CTG-TCT-CCA*-TCC-TCT-TCA*-CT (SEQ ID NO:77),

wherein A* represents nucleotides functionalized to incorporate a cholic acid functionality linked via a 2'-O-pentyl-amino linking group to the 2' position of the designated nucleotide. HPLC retention times are shown in Table 1 below.

D. Functionalization with Digoxigenin

1. Single Site Modification

A 10 O.D. unit (A_{260}) portion of Oligomer 12 (see, Example 1) was treated with digoxigenin-3-O-methylcarbonyl-ε-aminocaproic N-hydroxy succinimide ester (Boehringer Mannheim Corporation, Indianapolis, IN) in 200 μl of 0.1 M borate pH 8.3 buffer/40 μl DMF. The reaction mixture was let stand overnight. The product was isolated as per the method of Example 2(A)(1). Work up of the reaction gave the oligonucleotide:

Oligomer 25: 5'-CTG-TCT-CCA*-TCC-TCT-TCA-CT (SEQ ID NO:78),

wherein A* represents a nucleotide functionalized to incorporate a digoxigenin functionality linked via a 2'-O-pentyl-amino linking group to the 2' position of the designated nucleotide. HPLC retention times are shown in Table 1 below.

2. Multiple Site Modification

A 10 O.D. units (A_{260}) portion of Oligomer 13 (see, Example 1) was treated with digoxigenin-3-O-methylcarbonyl-ε-aminocaproic N-hydroxy succinimide ester (Boehringer Mannheim Corporation, Indianapolis, IN) in

300 μl of 0.1 M borate pH 8.3 buffer/50 μl DMF. The reaction mixture was let stand overnight. The product was isolated as per the method of Example 2(A)(1). Work up as per Example 2(A)(1) gave the oligonucleotide:

Oligomer 26: 5'-CTG-TCT-CCA*-TCC-TCT-TCA*-CT (SEQ ID NO:77),

wherein A* represents nucleotides functionalized to incorporate a cholic acid functionality linked via a 2'-O-pentyl-amino linking group to the 2' position of the designated nucleotide. HPLC retention times are shown in Table 1 below.

TABLE 1:

HPLC RETENTION TIMES OF OLIGONUCLEOTIDES FUNCTIONALIZED AT THE 2' POSITION

Oligomer	Retention Time (Minutes) Mono Substitution	Multiple Substitution	
Oligomer 12[1]	21.78		
Oligomer 13[1]		22.50	
Oligomer 19[2]	23.58		
Oligomer 20[2]		24.16[a]	25.19[b]
Oligomer 21[3]	26.65		
Oligomer 22[3]		26.99[a]	29.33[b]
		27.55[a]	
Oligomer 23[4]	30.10		
Oligomer 24[4]		30.38[a]	37.00[b]
		32.22[a]	
Oligomer 25[5]	28.06		
Oligomer 26[5]		28.14[a]	33.32[b]
		29.24[a]	

Conditions: Waters 600E with 991 detector, Hamilton PRP-1 column 0.7 x 15 cm; solvent A: 50 mM TEAA pH 7.0; B: 45 mM TEAA with 80% acetonitrile: 1.5 ml flow rate: Gradient: 5% B for first 5 minutes, linear (1%) increase in B every minute thereafter;

[a] Mono conjugated minor product;

[b] Doubly conjugated major product;

[1] Parent Oligonucleotide - no 2' functionalization;

[2] 2' Biotin functionalization;
[3] 2' Fluorescein functionalization;
[4] 2' Cholic Acid functionalization; and
[5] 2' Digoxigenin functionalization.

EXAMPLE 3: CHARACTERIZATION OF FUNCTIONALIZED OLIGONUCLEOTIDES

PROCEDURE A: Confirmation of Structure of Functionalized Oligonucleotides Containing a Tethered 2'-Amino Moiety

Oligonucleotides of the invention were digested with snake venom phosphodiesterase and calf-intestine alkaline phosphatase to their individual nucleosides. After digestion, the nucleoside composition was analyzed by HPLC. The HPLC analysis established that functionalized nucleotide compounds having the tethered 2'-amino moiety thereon were correctly incorporated into the oligonucleotide.

Snake venom phosphodiesterase [Boehringer-Mannheim cat. #108260, 1 mg (1.5 units)/0.5 ml] and alkaline phosphatase from calf intestine (1 unit/microliter, Boehringer-Mannheim cat. # 713023) in Tris-HCl buffer (pH 7.2, 50 mM) were used to digest the oligonucleotides to their component nucleosides. To 0.5 O.D. units of oligonucleotide in 50 μl buffer (nearly 40 μM final concentration for a 20 mer) was added 5 μl of snake venom phosphodiesterase (nearly 0.3 units/mL, final concentration) and 10 μl of alkaline phosphatase (app. 150 units/mL, final concentration). The reaction mixture was incubated at 37°C for 3 hours. Following incubation, the reaction mixture was analyzed by HPLC using a reverse phase analytical column (app. 30 x 2.5 cm); solvent A: 50 mM TEAA pH 7; solvent B: acetonitrile; gradient 100% for 10 minutes, then 5% B for 15 minutes, then 10% B and then

wash. The results of these digestion are shown in Table 2 for representative oligonucleotides.

TABLE 2:
OLIGONUCLEOTIDE ANALYSIS VIA ENZYMATIC DIGESTION

Oligomer	Abs. max.	Observed Ratios** 267 C	252 G	267 T	260 A*	260 A
Oligomer 10		2	1		1	
Oligomer 11		3	5	2	1	
Oligomer 12		9	1	8	1	1
Oligomer 13		9	1	8	2	

* Nucleoside having 2'-O-linker attached thereto; and

** Corrected to whole numbers.

As is evident from comparing the results in Table 2 to the Oligomer structures (*see*, Example 1), the correct nucleoside ratios are observed for the component nucleotides of the test oligonucleotides.

PROCEDURE B: Determination of Melting Temperatures (Tm's) of Cholic Acid Oligonucleotide Conjugates

The relative ability of oligonucleotides to bind to their complementary strand is compared by determining the melting temperature of the hybridization complex of the oligonucleotide and its complementary strand. The melting temperature (Tm), a characteristic physical property of double helices, denotes the temperature in degrees centigrade at which 50% helical versus coil (un-hybridized) forms are present. Tm is measured by using the UV spectrum to determine the formation and breakdown (melting) of hybridization. Base stacking, which occurs during hybridization, is accompanied by a reduction in UV absorption (hypochromicity). Consequently, a reduction

in UV absorption indicates a higher T_m. The higher the Tm, the greater the strength of the binding of the strands. Non-Watson-Crick base pairing has a strong destabilizing effect on the Tm. Consequently, absolute fidelity of base pairing is necessary to have optimal binding of an antisense oligonucleotide to its targeted RNA.

1. Terminal End Conjugates

a. Synthesis

A series of oligonucleotides were synthesized utilizing standard synthetic procedures (for un-functionalized oligonucleotides) or the procedure of Example 3(A) in U.S. Patent 6,265,558 for oligonucleotides having a 5'-terminus amino linker bearing oligonucleotide or the procedure of Example 3(B) in U.S. Patent 6,265,558 for 5'-terminus cholic acid-bearing oligonucleotides. Each of the oligonucleotides had the following 5-LO antisense sequence: 5'-TCC-AGG-TGT-CCG-CAT-C-3' (SEQ ID NO:6). The nucleotides were synthesized on a 1.0 μmol scale. Oligomer 32 was the parent compound having normal phosphodiester inter-nucleotide linkages. Oligomer 33 incorporated phosphorothioate inter-nucleotide linkages in the basic oligonucleotide sequence. Oligomer 34 is a an intermediate oligonucleotide having a 5'-aminolink at the 5'-terminus of the basic oligonucleotide sequence and Oligomer 35 was a similar 5'-aminolink compound incorporating phosphorothioate inter-nucleotide linkages. Oligomer 36 is a 5'-terminus cholic acid conjugate of the basic phosphodiester oligonucleotide sequence while Oligomer 37 is a similar 5'-cholic acid conjugate incor-porating phosphorothioate inter-nucleotide linkages.

Oligomers 32 and 33 were synthesized in a "Trityl-On" mode and were purified by HPLC. Oligomers 34 and 35 were synthesized as per Example 3(A) in U.S. Patent 6,265,558 without or with Beaucage reagent treatment, to yield phosphodiester or phosphorothioate inter-nucleotide linkages, respectively. Oligomers 36 and 37 were prepared from samples of Oligomers 34 and 35, respectively, utilizing a solution of cholic acid N-hydroxysuccinimide ester (Compound 1) 1 dissolved in DMF as per Example 3(B) in U.S. Patent 6,265,558. Oligomers 36 and 37 were purified by HPLC. The products were concentrated and desalted in a Sephadex G-25 column. Gel electrophoresis analyses also confirmed a pure product with the pure conjugate moving slower than the parent oligonucleotide or 5'-amino functionalized oligonucleotide.

b. Melting Analysis

The test oligonucleotides (either the phosphodiester, phosphorothioate, cholic acid conjugated phosphodiester, cholic acid conjugated phosphorothioate or 5'-aminolink intermediate phosphodiester or phosphorothioate oligonucleotides of the invention or otherwise) and either the complementary DNA or RNA oligonucleotides were incubated at a standard concentration of 4 μM for each oligonucleotide in buffer (100 mM NaCl, 10 mM Na-phosphate, pH 7.0, 0.1 mM EDTA). Samples were heated to 90 degrees C and the initial absorbance taken using a Guilford Response II spectrophotometer (Corning, Inc., Corning, NY). Samples were then slowly cooled to 15°C and then the change in absorbance at 260 nm was monitored during the heat

denaturation procedure. The temperature was elevated 1 degree/absorbance reading and the denaturation profile analyzed by taking the 1st derivative of the melting curve. Data was also analyzed using a two-state linear regression analysis to determine the Tm's. The results of these tests are shown in Table 3 as are the HPLC retention times of certain of the test compounds.

TABLE 3:

MELTING TEMPERATURE OF THE HYBRIDIZATION COMPLEX OF THE OLIGONUCLEOTIDE AND ITS COMPLEMENTARY STRAND

Oligomer No.	Tm**		HPLC Ret. Time* (min.)
	DNA	RNA	
32	62.6	62.0	----
33	55.4	54.9	----
34	ND	ND	13.6
35	ND	ND	17.0
36	63.4	62.4	22.0
37	56.3	55.8	22.5

* HPLC conditions: Walters Delta Pak C-18 RP 2.5u column; at 0 min 100% 0.1 TEAA; at 30 min 50% TEAA and 50% Acetonitrile: Flow rate 1.0 ml/min.

** Tm at 4μM each strand from fit of duplicate melting curves to 2-state model with linear sloping base line. Conditions: 100 mM NaCl, 10 mM Phosphate, 0.1 mM EDTA, pH 7.0.

ND = not determined

As is evident from the data presented in Table 3, conjugation of cholic acid at the end of the oligonucleotides does not affect the Tm of the oligonucleotides.

2. Strands Incorporating 2'-O-Pentylamino Linker

a. Synthesis

An oligonucleotide of the sequence GGA*-CCG-GA*A*-GGT-A*CG-A*G (Oligomer 38, SEQ ID NO:7), wherein A*

represents a nucleotide functionalized to incorporate a pentylamino functionality at its 2'-position, was synthesized in a one micromole scale utilizing the method of Example 1(B). The oligonucleotide was purified by reverse phase HPLC, detritylated and desalted on Sephadex G-25. PAGE gel analysis showed a single band. A further oligonucleotide, Oligomer 39, having the same sequence but without any 2'-O-amino linker was synthesis in a standard manner. A complementary DNA oligonucleotide of the sequence 5'-CCT-GGC-CTT-CCA-TGC-TC (Oligomer 40, SEQ ID NO:8) was also synthesized in a standard manner as was a complementary RNA oligonucleotide of the sequence 5'-CCU-GGC-CUU-CCA-UGC-UC (Oligomer 41, SEQ ID NO:9).

b. Melting Analysis

Melting analysis was conducted as per the method of Procedure B(1)(b). The results are shown in Table 4.

TABLE 4:
MELTING TEMPERATURE OF THE HYBRIDIZATION COMPLEX OF THE OLIGONUCLEOTIDE AND ITS COMPLEMENTARY STRAND

Oligomer No.	Tm*	
	DNA[1]	RNA[2]
38	54.5	58.0
39	60.6	56.9
(Δ_{38-39})	(6.1)	(1.1)

* Tm at 4μM each strand from fit of duplicate melting curves to 2-state model with linear sloping base line. Conditions: 100 mM NaCl, 10 mM Phosphate, 0.1 mM EDTA, pH 7.0.
[1] Against DNA complementary strand, Oligomer 40.
[2] Against RNA complementary strand, Oligomer 41

As is evident from Table 4, against the RNA complementary strand the change in Tm's between the strand having 2'-amino linkers thereon and the unmodified strand is 1.1 degrees (0.22 change per modification). Against the DNA strand, the change is -6.1 degrees (-1.2 change per modification). When compared to the parent unmodified oligonucleotide the 2'-amino linker-containing strand has a stabilizing effect upon hybridization with RNA and a destabilizing effect upon hybridization with DNA.

Compounds of the invention were tested for their ability to increase cellular uptake. This was determined by judging either their ability to inhibit the expression of bovine papilloma virus-1 (BPV-1) or an assay involving luciferase production (for HIV-1).

PROCEDURE C: Determination of Cellular Uptake Judged By The Inhibition Of Expression of Bovine Papilloma Virus-1 (bpv-1) As Measured By an E2 Transactivation Assay

For this test, a phosphorothioate oligonucleotide analog of the sequence 5'-CTG-TCT-CCA-TCC-TCT-TCA-CT (Oligomer 42, SEQ ID NO:72) was used as the basic sequence. This sequence is designed to be complementary to the translation initiation region of the E2 gene of bovine papilloma virus type 1 (BPV-1). Oligomer 42 served as the positive control and standard for the assay. Oligomer 3 (from Example 4 in U.S. Patent 6,265,558) served as a second test compound. It has the same nucleobase sequence as Oligomer 42 but is a phosphorothioate oligonucleotide and, further, has a cholic acid moiety conjugated at the 3'-end of the oligonucleotide. Oligomer 2 (from Example 2 in U.S. Patent 6,265,558) served as a third test compound. It is also of the same sequence and is a phosphorothioate oligonucleotide, but has a cholic acid moiety conjugated at its 5'-end. Oligomer 5 (from Example 5 in U.S. Patent No. 6,265,558) served as a fourth test compound. It also has the same nucleobase sequence and is a phosphorothioate oligonucleotide, but has cholic acid moieties conjugated at both its 3'- and 5'-ends. Compounds five, six and seven served as negative controls for the assay. The fifth test compound was a phosphorothioate oligonucleotide with no significant sequence homology with BPV-1. The sixth test compound was a further phosphorothioate oligonucleotide with no significant sequence homology with BPV-1. The seventh test compound, was a phosphorothioate oligonucleotide with cholic acid conjugated to the 3'-end but having no significant sequence homology with BPV-1.

For each test I-38 cells were plated at $5x10^4$ cells per cm^2 in 60 mm petri dishes. Eight hours after plating, medium was aspirated and replaced with medium containing

the test oligonucleotide and incubated overnight. Following incubation, medium was aspirated and replaced with fresh medium without oligonucleotide and incubated for one hour. Cells were then transfected by the $CaPO_4$ method with 2 μg of pE2RE-1-CAT. After a four hour incubation period cells were glycerol shocked (15% glycerol) for 1 minute followed by washing 2 times with PBS. Medium was replaced with DMEM containing oligo-nucleotide at the original concentration. Cells were incubated for 48 hours and harvested. Cell lysates were analyzed for chloramphenicol acetyl transferase by standard procedures. Acetylated and nonacetylated ^{14}C-chloramphenicol were separated by thin layer chromatography and quantitated by liquid scintillation. The results are expressed as percent acetylation.

Two lots of the positive control compound were found to acetylate at a level of 29% and 30%. The negative controls, test compounds five, six and seven, were found to acetylate at 59%, 58% and 47%, respectively. The 3'-cholic acid conjugate test compound, Oligomer 3, was found to acetylate to 23%, the 5'-cholic acid conjugate test compound, Oligomer 2, was found to acetylate to 36% and the test compound conjugated at both the 3'-end and the 5'-end, Oligomer 5, was found to acetylate to 27%.

The results of this test suggest that placement of a cholic acid moiety at the 3'-terminus of an oligonucleotide increase the activity. This in turn suggests that the increased activity was the result of increased cellular membrane transport.

PROCEDURE D: Determination of Cellular Uptake Judged By Inhibition of pHIVluc With Cholic Acid Linked 2'-O-Methyl Substituted Oligonucleotides

[redacted]

For this test the absence of an oligonucleotide in a test well served as the control. All oligonucleotides were tested as 2'-O-methyl analogs. For this test an oligonucleotide of the sequence 5'-CCC-AGG-CUC-AGA (Oligomer 43, SEQ ID NO:10), where each of the nucleotides of the oligonucleotide includes a 2'-O-methyl substituent group served as the basic test compound.

The second test compound, 5'-CHA-CCC-AGG-CUC-AGA (Oligomer 44, SEQ ID NO:10), wherein CHA represents cholic acid and where each of the nucleotides of the oligonucleotide includes a 2'-O-methyl substituent group, was also of the same sequence as the first test compound. This second test compound included cholic acid conjugated to its 5'-end and was prepared as per the method of Example 3 in U.S. Patent 6,265,558 utilizing 2'-O-methyl phosphoramidite intermediates as identified in Example 1(C).

The third test compound, 5'-CCC-AGG-CUC-AGA-3'-CHA (Oligomer 45, SEQ ID NO:10), wherein CHA represents cholic acid and where each of the nucleotides of the oligonucleotide includes a 2'-O-methyl substituent group, was also of the same sequence as the first test compound. The third test compound included cholic acid conjugated to its 3'-end and was prepared as per the method of Example 4 in U.S. Patent 6,265,558 utilizing 2'-O-methyl phosphoramidite intermediates as identified in Example 1(C).

The fourth test compound was a 2'-O-Me oligonucleotide of a second sequence, 5'-GAG-CUC-CCA-GGC (Oligomer 46, SEQ ID NO:11), where each of the nucleotides of the oligonucleotide includes a 2'-O-methyl substituent group.

The fifth test compound was of sequence 5'-CHA-GAG-CUC-CCA-GGC (Oligomer 47, SEQ ID NO:11), wherein CHA represents cholic acid and where each of the nucleotides of the oligonucleotide includes a 2'-O-methyl substituent group. It was of the same sequence as the fourth test compound. This test compound included cholic acid conjugated to its 5'-end and was prepared as per the method of Example 3 in U.S. Patent 6,265,558 utilizing 2'-O-methyl phosphoramidite intermediates as identified in Example 1(C).

A sixth test compound was a randomized oligonucleotide of the sequence 5'-CAU-GCU-GCA-GCC (Oligomer 48, SEQ ID NO:12).

HeLa cells were seeded at $4x10^5$ cells per well in 6-well culture dishes. Test oligonucleotides were added to triplicate wells at 1 μM and allowed to incubate at 37°C for 20 hours. Medium and oligonucleotide were then removed, cells washed with PBS and the cells were $CaPO_4$ transfected. Briefly, 5μg of pHIVluc, a plasmid expressing the luciferase cDNA under the transcriptional control of the HIV LTR constructed by ligating the KpnI/HindIII restriction fragments of the plasmids pT3/T7luc and pHIVpap (NAR 19(12)) containing the luciferase cDNA and the HIV LTR respectively, and 6 μg of pcDEBtat, a plasmid expressing the HIV tat protein under the control of the SV40 promoter, were added to 500 μl of 250 mM $CaCl_2$, then 500 μl of 2x HBS was added followed by vortexing. After 30 minutes, the $CaPO_4$ precipitate was divided evenly between the six wells of the plate, which was then incubated for 4 hours. The media and precipitate were then removed, the cells washed with PBS, and fresh oligonucleotide and media were added. Incubation was continued overnight. Luciferase activity

was determined for each well the following morning. Media was removed, then the cells washed 2X with PBS. The cells were then lysed on the plate with 200 μl of LB (1% Trit X-100, 25 mM Glycylglycine pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1mM DTT). A 75 μl aliquot from each well was then added to a well of a 96 well plate along with 75 μl of assay buffer (25 mM Glycylglycine pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM KPO_4, 1 mM DTT, 2.5 mM ATP). The plate was then read in a Dynatec multiwell luminometer that injected 75 μl of Luciferin buffer (25 mM Glycylglycine pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 4 mM DTT, 1 mM luciferin) into each well, immediately reading the light emitted (light units).

The random sequence compound (Oligomer 48) and the other non-cholic acid-conjugated test compounds (Oligomers 43 and 46) had comparable activity. The 5'-conjugate of the first sequence (Oligomer 44) also had activity comparable to the non-conjugated compounds. The 5'-conjugate of the second sequence (Oligomer 47) showed a three-fold increase in activity compared to the non-conjugated compounds and the 3'-conjugate of the first sequence (Oligomer 45) showed a further 3-fold increase in activity compared to Oligomer 47.

All the test cholic acid-bearing oligonucleotides showed significant inhibition of luciferase production compared to non-cholic acid- bearing oligonucleotides. This suggests that the increased activity was the result of increased cellular membrane transport of the cholic acid-bearing test oligonucleotides.

EXAMPLE 4: PREPARATION OF MODIFIED NUCLEOSIDES AND NUCLEOTIDES, AND PHOSPHORAMIDITE AND CONTROLLED PORE GLASS (CPG) DERIVATIVES THEREOF

A. Preparation of 5'-O-[Dimethoxytrityl]-2'-O-[hexyl-(Ω-N-phthalimido)amino]uridine and 5'-O-[dimethoxytrityl]-3'-O-[hexyl(Ω-N-phthalimidoamino)uridine.

2',3'-*O*-Dibutyl stannylene-uridine was synthesized according to the procedure of Wagner *et. al.* (*J. Org. Chem.*, 1974, *39*, 24). This compound was dried over P_2O_5 under vacuum for 12 hours. To a solution of this compound (29 g, 42.1 mmols) in 200 ml of anhydrous DMF were added (16.8 g, 55 mmols) of 6-bromohexyl phthalimide and 4.5 g of sodium iodide and the mixture was heated at 130°C for 16 hours under argon. The reaction mixture was evaporated, co-evaporated once with toluene and the gummy tar residue was applied on a silica column (500 g). The column was washed with 2L of ethyl acetate (EtOAc) followed by eluting with 10% methanol (MeOH):90% EtOAc. The product, 2'- and 3'-isomers of O-hexyl-Ω-N-phthalimido uridine, eluted as an inseparable mixture (R_f=0.64 in 10% MeOH in EtOAc). By ^{13}C NMR, the isomeric ratio was about 55% of the 2' isomer and about 45% of the 3' isomer. The combined yield was 9.2 g (46.2%). This mixture was dried under vacuum and re-evaporated twice with pyridine. It was dissolved in 150 mL anhydrous pyridine and treated with 7.5 g of dimethyocytrityl chloride (22.13 mmols) and 500 mg of dimethylaminopyridine (DMAP). After 2 hour, thin layer chromatography (TLC; 6:4 EtOAc:Hexane) indicated complete disappearance of the starting material and a good separation between 2' and 3' isomers (R_f=0.29 for the 2' isomer and 0.12 for the 3' isomer). The reaction mixture was quenched by the addition of 5mL of CH_3OH and evaporated under reduced pressure. The residue was dissolved in 300mL CH_2Cl_2, washed successively with saturated $NaHCO_3$ followed by saturated NaCl solution. It was dried over Mg_2SO_4 and evaporated to give 15 g of a brown foam which was purified on a silica gel (500 g) to give 6.5 g of the 2'-isomer and 3.5 g of the 3' isomer.

B. Preparation of 5'-O-Dimethoxytrityl-2'-O-[hexyl-(Ω-N-phthalimido)amino]uridine-3'-O-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidite

The 5'-dimethoxytrityl-2'-[O-hexyl-(Ω-N-phthalimido)-amino]uridine (4 g, 5.2 mmole) was dissolved in 40 mL of anhydrous CH_2Cl_2. To this solution diisopropylaminetetrazolide (0.5 g, 2.9 mmol) was added and stirred overnight. TLC (1:1 EtoAC/hexane) showed complete disappearance of starting material. The reaction mixture was transferred with CH_2Cl_2 and washed with saturated $NaHCO_3$ (100 mL) followed by saturated NaCl solution. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to yield 6.4 g of a crude product that was purified in a silica column (200 g) using 1:1 hexane/EtOAc to give 4.6 g (4.7 mmol, 90%) of the desired phosphoramidite.

C. Preparation of 5'-O-(Dimethoxytrityl)-3'-O-[hexyl-(Ω-N-phthalimido)amino]uridine-2'-O-succinyl-aminopropyl controlled pore glass

Succinylated and capped aminopropyl controlled pore glass (CPG; 500Å pore diameter, aminopropyl CPG, 1.0 grams prepared according to Damha *et. al.* (*Nucl. Acids Res.*, 1990, *18*, 3813.) was added to 12 ml anhydrous pyridine in a 100 ml round-bottom flask. 1-(3-Dimethylaminopropyl)-3-ethyl-carbodiimide (DEC; 0.38 grams, 2.0 mmol)], triethylamine (TEA; 100 μl, distilled over CaH_2), dimethylaminopyridine (DMAP; 0.012 grams, 0.1 mmol) and nucleoside 5'-O-dimethoxytrityl-3'-O-[hexyl-(Ω-N-phthalimidoamino)]uridine (0.6 grams, 0.77 mmol) were added under argon and the mixture shaken mechanically for 2 hours. More nucleoside (0.20 grams) was added and the mixture shaken an additional 24 hours. CPG was filtered

off and washed successively with dichloromethane, triethylamine, and dichloromethane. The CPG was then dried under vacuum, suspended in 10 ml piperidine and shaken 15 minutes. The CPG was filtered off, washed thoroughly with dichloromethane and again dried under vacuum. The extent of loading (determined by spectrophotometric assay of dimethoxytrityl cation in 0.3 M p-toluenesulfonic acid at 498 nm) was approximately 28 μmol/g. The 5'-O-(dimethoxytrityl)-3'-O-[hexyl-(Ω-N-phthalimidoamino]uridine-2'-O-succinyl-aminopropyl controlled pore glass was used to synthesize the oligomers 5'-GACU*-3' and 5'-GCC-TTT-CGC-GAC-CCA-ACA-CU*-3' (SEQ ID NO:13, where the * indicates the derivatized nucleotide) in an ABI 380B DNA synthesizer using phosphoramidite chemistry standard conditions. 45 and 200 O.D.'s of the 4-mer and 20-mer, respectively, were obtained from two and three 1 μmol syntheses after purification by RP-HPLC and desalting.

The oligomer 5'-GACU*-3' was used to confirm the structure of 3'-O-hexylamine tether introduced into the oligonucleotide by NMR. As expected a multiplet signal was observed between 1.0-1.8 ppm in ^{1}H NMR. The oligomer 5'-GCC-TTT-CGC-GAC-CCA-ACA-CU*-3' (SEQ ID NO:13) belongs to a HCV sequence and it was used to show the nuclease resistance properties of the 3'-O-amino tether (*see*, Example 5).

D. Preparation of 5'-O-(Dimethoxytrityl)-2'-O-[hexyl-(Ω-N-phthalimido)amino] 3'-O-succinyl-aminopropyl controlled pore glass

The procedure of Example 4(C) was repeated, except that 5'-O-(Dimethoxytrityl)-2'-O-[hexyl-(Ω-N-

phthalimidoamido)amino]uridine was used in the loading process.

E. Preparation of 5'-O-(Dimethoxytrityl)-2'-O-(hexylamino)-uridine

5'-O-(dimethoxytrityl)-2'-O-[hexyl-(Ω-N-phthalimido amino)]uridine (4.5 grams, 5.8 mmol) was dissolved in 200 ml methanol in a 500 ml flask. Hydrazine (1 ml, 31 mmol) was added to the stirring reaction mixture. The mixture was heated to 60-65°C in an oil bath and refluxed 14 hours. Solvent was evaporated *in vacuo*. The residue was dissolved in dichloromethane (250 ml) and extracted twice with an equal volume NH_4OH. The organic layer was evaporated to yield 4.36 grams of crude product, and NMR indicated that the product was not completely pure. R_f=0 in 100% ethyl acetate. The product was used in subsequent reactions without further purification.

F. Preparation of 5'-O-(dimethoxytrityl)-3'-O-[hexylamino] uridine

The procedure of Example 4(E) was repeated, except that 5'-O-(dimethoxytrityl)-3'-O-[hexyl-(Ω-N-phthalimido-amino)] uridine was used as the starting material.

G. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(1-pyrene propyl carbonyl)amino]uridine

5'-O-Dimethoxytrityl-2'-O-(hexylamino)uridine (0.5g, 0.78 mmol) was dissolved in anhydrous DMF (15 mL). 1-Hydroxybenzotriazole (0.16 grams, 1.17 mmol) and 1-pyrene-butyric acid pentafluorophenyl ester (0.53 grams, 1.17 mmol) were added to the reaction mixture. The mixture was stirred under argon at room temperature for 2 hours, after which it was concentrated *in vacuo*.

[redacted]

Residual DMF was coevaporated with toluene. The residue was dissolved in dichloromethane (50 mL) and washed with an equal volume saturated $NaHCO_3$. The aqueous layer was washed with dichloromethane and the combined organic extracts washed with an equal volume saturated NaCl. The aqueous layer was washed with dichloromethane and the combined organic layers dried over $MgSO_4$ and concentrated. The residue was chromatographed on a silica gel column, eluting with a gradient of 50% ethyl acetate in hexanes to 100% ethyl acetate. The desired product (0.83 grams, 58%) eluted with 100% ethyl acetate (R_f 0.46 by thin-layer chromatography (TLC)).

H. Preparation of 5'-O-[Dimethoxytrityl]-2'-O-[hexyl-N-(1-pyrene propyl carbonyl)amino]uridine-3'-O-(2-cyanoethyl-N, N-diisopropyl)phosphoramidite

5'-O-[Dimethoxytrityl]-2'-O-[hexyl-N-(1-pyrene propyl carbonyl)amino] uridine (0.80 grams, 0.87 mmol) was dissolved in 20 mL dry dichloromethane. 2-Cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (purchased from Sigma Chemical Co; 800 μL, 2.4 mmol) and diisopropylamine tetrazolide (0.090 grams, 0.52 mmol) were added to the mixture, which was stirred under argon for 20 hours The reaction mixture was then concentrated *in vacuo* and the residue dissolved in dichloromethane (75 mL). The solution was washed with an equal volume of saturated $NaHCO_3$. The aqueous layer was washed with dichloromethane (20 mL) and the combined organic layers washed with an equal volume of saturated NaCl. The aqueous layer was washed with dichloromethane (20 mL) and the combined organic layers dried over $MgSO_4$ and concentrated. The residue was chromatographed on a silica gel column, eluting with a gradient of 50% ethyl acetate in hexanes to 100% ethyl acetate. The desired

product (750 mg, 78% yield, R_f 0.54 by TLC in 100% ethyl acetate) eluted with 100% ethyl acetate.

G. Preparation of 2'-O-[hexyl-N-(1-pyrene-propyl-carbonyl) amino] uridine

5'-O-dimethoxytrityl-2'-O-[hexyl-N-(1-pyrene-propyl-carbonyl)amino]uridine (1.0 g) was dissolved in 20 mL CH_2Cl_2 and kept in ice-bath for 10 minutes. To the cold solution, 5 mL of 80% acetic acid in water was added and the solution was left to stand for 30 minutes. It was then evaporated to dryness and loaded into a silica column and eluted with 10% methanol in methylene chloride to give 2'-O-[hexyl-N-(1-pyrene-propyl-carbonyl)amino]uridine.

J. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(1-pyrene propyl carbonyl)amino]uridine-3'-O-[succinylaminopropyl]-controlled pore glass

Succinylated/capped aminopropyl controlled pore glass was dried under vacuum for 3 hours immediately before use. A portion (0.3 g) was added to 3 ml anhydrous pyridine in a 50 ml round-bottom flask. DEC (0.12 grams, 0.63 mmol), TEA (25 μl, distilled over CaH_2), DMAP (0.005 grams, mmol) and 5'-O-(dimethoxytrityl)-3'-O-[hexyl-N-(1-pyrene propyl carbonyl] amino]uridine (0.21 grams, 0.22 mmol) were added under argon and the mixture shaken mechanically for 19 hours. More nucleoside (0.025 grams) was added and the mixture shaken an additional 5.5 hours. Pentachlorophenol (0.045 grams, mmol) was added and the mixture shaken 18 hours. CPG was filtered off and washed successively with dichloromethane, triethylamine, and dichloromethane. The resulting CPG was then dried under vacuum, suspended in 15 ml piperidine and shaken 30 minutes. CPG was filtered off,

washed thoroughly with dichloromethane and again dried under vacuum. The extent of loading (determined by spectrophotometric assay of dimethoxytrityl cation in 0.3 M p-toluenesulfonic acid at 498 nm) was approximately 27 μmol/g. The product solid support was subsequently used to synthesize the oligomers.

K. Preparation of 5'-O-dimethoxytrityl-3'-O-[hexyl-N-(1-pyrene propyl carbonyl] amino] uridine-2'-O-(succinyl amino propyl) controlled pore glass

The procedure of Example 4(J) is repeated, except that 5'-O-dimethoxytrityl-3'-O-[hexyl-N-(1-pyrene propyl carbonyl] amino] uridine is used.

L. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(5-thio carbonyl-3,6-dipivolyl-fluorescein)amino]uridine

Fluorescein isothiocyanate (Isomer I, available from Cal Biochem, La Jolla, CA) was treated with 12 equivalents of pivolyl chloride in Et_3N/THF to give di-O-pivolyl fluorescein isothiocyanate. This compound was purified in silica gel column using 3:1 hexane:ethyl acetate. Nucleoside 5'-O-(dimethoxytrityl)-2'-O-(hexylamino)uridine was then condensed with dipivolyl fluorescein isothiocyanate in CH_2Cl_2/pyrimidine. The resultant compound 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(5-thiocarbonyl-3,6-dipivolyl-fluorescein)amino]uridine is then purified by using 100% ethyl acetate, in a silica column.

M. Preparation of 5'-O-dimethoxytrityl-2'-O-[hexyl-N-(5-thiocarbonyl-3,6-di-pivolyl fluorescein) amino] uridine-3'-O-(2-cyanoethyl, N-N-diisopropyl phosphoramidite

[redacted]

5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(5-thiocarbonyl-3,6-dipivolyl fluorescein)amino]uridine (0.75 grams, 0.672 mmol) was dissolved in dry dichloromethane (20 mL). 2-Cyanoethyl *N,N,N',N'*-tetraisopropyl-phosphorodiamidite (700 μL, 2.2 mmol) and diisopropylamine tetrazolide were added to the mixture, which was stirred under argon for 16 hours. The reaction mixture was then concentrated *in vacuo* and the residue dissolved in dichloromethane (75 mL) followed by washing with an equal volume of saturated $NaHCO_3$. The aqueous layer was washed with dichloromethane (50 mL) and the combined organic layers washed with an equal volume of saturated NaCl. The aqueous layer was washed with dichloromethane (50 mL) and the combined organic layers dried over $MgSO_4$ and concentrated. The residue was chromatographed on a silica gel column, eluting with a gradient of 25% ethyl acetate in hexanes to 100% ethyl acetate. The desired product (670 mg, 77% yield, R_f 0.79 by TLC) eluted with 100% ethyl acetate.

N. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(5-thiocarbonyl-3,6-di-pivolyl fluorescein)amino]uridine-3'-O-(succinylaminopropyl) controlled pore glass

Succinylated and capped aminopropyl controlled pore glass (CPG) is dried under vacuum for 3 hours immediately before use. CPG (0.3 grams) is added to 3 ml anhydrous pyridine in a 50 ml round-bottom flask. DEC (0.12 grams, 0.63 mmol), TEA (25 μl, distilled over CaH_2, DMAP (dimethyl amino pyridine) (0.005 grams, 0.04 mmol) and 5'-O-dimethoxytrityl-2'-O-[hexyl-N-(5-thiocarbonyl-3,6-di-pivolyl fluorescein) amino] uridine (0.21 grams, 0.19 mmol) are added under argon and the mixture shaken mechanically for 19 hours. More nucleoside (0.025 grams)

is added and the mixture shaken an additional 5.5 hours. Pentachlorophenol (0.045 grams, 0.17 mmol) is added and the mixture shaken 18 hours. CPG is filtered off and washed successively with dichloromethane, triethylamine, and dichloromethane. CPG then is dried under vacuum, suspended in 15 mL piperidine and shaken 30 minutes. CPG is filtered off, washed thoroughly with dichloromethane, and again dried under vacuum. The extent of loading is then determined by spectrophotometric assay of dimethoxytrityl cation in 0.3 M p-toluenesulfonic acid at 498 nm.

O. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(3-oxycarbonyl-cholesteryl)amino]uridine

Nucleoside 5'-O-(dimethoxytrityl)-2'-O-[hexylamino]-uridine (3.85 g, 6.0 mmol) was dissolved in anhydrous pyridine/dichloromethane 50/50 (v/v) (20 mL). Cholesteryl chloroformate (Fluka, 3.0 g, 6.68 mmol) was dissolved in anhydrous dichloromehthane (20 ml) and added slowly under argon with a syringe to the stirring reaction mixture. The mixture was stirred under argon at room temperature for 2 h after which it was concentrated *in vacuo*. Residual DMF was coevaporated with toluene. The residue was dissolved in dichloromethane (50 mL) and washed with an equal volume saturated $NaHCO_3$. The aqueous layer was washed with dichloromethane and the combined organic extracts washed with an equal volume saturated NaCl. The aqueous layer was washed with dichloromethane and the combined organic layers dried over $MgSO_4$ and concentrated. The residue was chromatographed on a silica gel column with a gradient of 25% ethyl acetate in hexanes to 100% ethyl acetate. The desired product (3.78 g, 60%) eluted with 100% ethyl acetate (R_f 0.41 by TLC).

P. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(3-oxycarbonyl-cholesteryl)amino]uridine-3'-O-[2-cyanoethyl-N,N-diisopropyl]phosphoramidite

Nucleoside 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(3-oxycarbonyl-cholesteryl)amino]uridine (3.44 g, 3.3 mmol) was dissolved in dry dichloromethane (75 mL). 2-cyanoethyl *N,N,N'N'*-tetraisopropylphosphorodiamidite (Sigma, 2.1 ml, 6.6 mmol) and diisopropylamine tetrazolide (0.29 g, 1.7 mmol) were added to the mixture, which was stirred under argon for 16 H. Dichloromethane (75 mL) was added to the solution, which was washed with an equal volume of saturated $NaHCO_3$. The aqueous layer was washed with an equal volume of dichloromethane. The aqueous layer was washed with dichloromethane (30 ml) and the combined organic layers washed with an equal volume of saturated NaCl. The aqueous layer was washed with dichloromethane (30 mL) and the combined organic layers dried over Mg_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on a silica gel column with a gradient of 25% ethyl acetate in hexanes to 70% ethyl acetate. The desired product (3.35 g, 82% yield, R_f=0.71 by TLC in 50% ethyl acetate in hexanes) eluted with 50% ethyl acetate.

Q. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(3-oxycarbonyl-cholesteryl)amino]uridine-3'-O-(succinyl aminopropyl)-controlled pore glass

Succinylated and capped controlled pore glass (0.3 grams) is added to 2.5 ml anhydrous pyridine in a 15 ml pear-shaped flask. DEC (0.07 grams, 0.36 mmol), TEA (100 μl, distilled over CaH_2), DMAP (0.002 grams, 0.016 mmol) and 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(3-oxycarbonyl-cholesteryl)amino]uridine (0.25 grams, 0.23 mmol) are added under argon and the mixture shaken mechanically for

16 hours. More nucleoside (0.20 grams) is added and the mixture shaken an additional 18 hours. Pentachlorophenol (0.03 grams, 0.11 mmol) is added and the mixture shaken 9 hours. CPG is filtered off and washed successively with dichloromethane, triethylamine, and dichloromethane. CPG is then dried under vacuum, suspended in 10 ml piperidine and shaken 15 minutes. CPG is filtered off, washed thoroughly with dichloromethane and again dried under vacuum. The extent of loading is determined by spectrophotometric assay of dimethoxytrityl cation in 0.3 M p-toluenesulfonic acid at 498 nm as approximately 39 μmol/g.

R. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(2,4-dinitrophenyl)amino]uridine

5'-O-(dimethoxytrityl)-2'-O-(hexylamino)uridine (0.88 grams, 1.37 mmol) was dissolved in methanol (20 mL). 2,4-Dinitrofluorobenzene (DNFB, 0.25 grams, 1.37 mmol) was added and the mixture shaken on a mechanical shaker. The reaction was monitored by TLC. After 90 minutes, another 0.25 grams of DNFB was added and the reaction mixture shaken an additional 30 minutes, followed by addition of another 0.25 grams of DNFB. After shaking 2.5 hours, the mixture was concentrated *in vacuo* and chromatographed on a silica gel column, eluting with a gradient of 25% ethyl acetate in hexanes to 100% ethyl acetate. The desired product (0.51 grams, 46%) eluted with 100% ethyl acetate (R_f 0.85 by TLC).

S. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(2,4-dinitrophenyl)amino]uridine-3'-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite

5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(2,4-dinitrophenyl)amino]uridine (0.45 grams, 0.55 mmol) was

dissolved in dry dichloromethane (12 mL). 2-Cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (380 μL, 1.2 mmol) and diisopropylamine tetrazolide (0.041 grams, 0.024 mmol) were added to the mixture, which was stirred under argon for 16 hours. The reaction mixture was then concentrated *in vacuo* and the residue dissolved in dichloromethane (75 mL) followed by washing with an equal volume of saturated $NaHCO_3$. The aqueous layer was washed with dichloromethane (25 mL) and the combined organic layers washed with an equal volume of saturated NaCl. The aqueous layer was washed with dichloromethane (25 mL) and the combined organic layers dried over $MgSO_4$ and concentrated. The residue was chromatographed on a silica gel column, eluting with a gradient of 20% ethyl acetate in hexanes to 100% ethyl acetate. The desired product (510 mg foam, 93% yield, R_f 0.70 by TLC) eluted with 100% ethyl acetate. ^{31}PNMR ($CDCl_3$): 150.56 and 150.82 ppm.

T. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(2,4-dinitrophenyl)amino]uridine-3'-O-(succinyl aminopropyl) controlled pore glass

Succinylated and capped controlled pore glass (0.3 grams) is added to 3 ml anhydrous pyridine in a 50 ml round-bottom flask. DEC (0.12 grams, mmol), TEA (25 μl, distilled over CaH_2), DMAP (0.005 grams, 0.041 mmol) and 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(2,4-dinitrophenyl)-amino]uridine (0.21 grams, 0.26 mmol) are added under argon and the mixture shaken mechanically for 19 hours. More nucleoside (0.025 grams) is added and the mixture shaken an additional 5.5 hours. Pentachlorophenol (0.045 grams, 0.16 mmol) is added and the mixture shaken 18 hours. CPG is filtered off and washed successively with dichloromethane, triethylamine, and dichloromethane. CPG then is dried under vacuum, suspended in 15 ml piperidine and shaken for 15 minutes. CPG is filtered off, washed thoroughly with dichloromethane, and again dried under vacuum. The extent of loading is determined by spectrophotometric assay of dimethoxytrityl cation in 0.3 M p-toluenesulfonic acid at 498 nm, as approximately 29 μmol/gm.

U. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(Nα-Nimid-Di-FMOC-L-Histidyl)amino]uridine

Nucleoside 5'-O-(dimethoxytrityl)-2'-O-(hexylamino)-uridine (0.97 g, 1.51 mmol) was dissolved in dichloro-methane (25 mL) and cooled to 0°C in an ice bath. Nα,Nimid-Di-FMOC-L-histine pentafluorophenyl ester (2.4 g, 3.1 mmol, purchased from Sigma) and 1-hydroxybenzotriazole (0.32 g, 0.24 mmol, purchased from Fluka) were added to the stirred reaction mixture stirred under argon. After 15 minutes, the ice bath was removed

and the mixture stirred under argon at room temperature for 72 h. The mixture was concentrated *in vacuo* and chromatographed on a silica gel column, eluting with a gradient of 50% ethyl acetate in hexanes to 70% ethyl acetate in hexanes. The desired product (0.53 g, 28%) eluted with 70% ethyl acetate (R_f 0.53 by TLC in 100% ethyl acetate).

V. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(Nα-Nimid-Di-FMOC-L-histidyl)-amino]-uridine-3'-O-[2-cyanoethyl-N,N-diisopropyl]phosphoramidite

5'-O-Dimethoxytrityl-2'-O-[hexyl-N-(Nα-Nimid-Di-FMOC-L-histidyl)amino]uridine (1.9 g, 1.6 mmol) is dissolved in dry dichloromethane (20mL). 2-Cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (800 μL, 2.4 mmol) and diisopropylamine tetrazolide (0.090 grams, 0.52 mmol) are added to the mixture, which is stirred under argon for 20 hours. The reaction mixture then is concentrated *in vacuo* and the residue dissolved in dichloromethane (75 mL). The solution is washed with an equal volume of saturated $NaHCO_3$. The aqueous layer is washed with dichloromethane (20 mL) and the combined organic layers washed with an equal volume of saturated NaCl. The aqueous layer is washed with dichloromethane (20 mL) and the combined organic layers dried over $MgSO_4$ and concentrated. The residue is chromatographed on a silica gel column, eluting with a gradient of 50% ethyl acetate in hexanes to 100% ethyl acetate. The desired product elutes with 100% ethyl acetate.

W. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(Nα-Nimid-Di-FMOC)-L-histidyl)amino]uridine-3'-O-[succinylaminopropyl] controlled pore glass

[redacted]

Succinylated and capped controlled pore glass (dried under vacuum for 3 hours immediately before use; 0.3 grams) is added to 3 ml anhydrous pyridine in a 50 ml round-bottom flask. DEC (0.12 grams, 0.63 mmol), TEA (25 μl, distilled over CaH_2), DMAP (0.005 grams, 0.04 mmol) and 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(Nα-Nimid-Di-FMOC)-L-histidyl)amino]uridine (0.21 grams, 0.17 mmol) are added under argon and the mixture shaken mechanically for 19 hours. More nucleoside (0.025 grams) is added and the mixture shaken an additional 5.5 hours. Pentachlorophenol (0.045 grams, 0.17 mmol) is added and the mixture shaken 18 hours. CPG is filtered off and washed successively with dichloromethane, triethylamine, and dichloromethane. CPG then is dried under vacuum, suspended in 15 ml piperidine and shaken 15 minutes. CPG is filtered off, washed thoroughly with dichloromethane and again dried under vacuum. The extent of loading is determined by spectrophotometric assay of dimethoxytrityl cation in 0.3 M p-toluenesulfonic acid at 498 nm. to be approximately 27 μmol/g.

X. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(Ω-methyl-polyethylene glycol-propionoyl)amino]uridine

Nucleoside 5'-O-(dimethoxytrityl)-2'-O-[hexylamino]-uridine, (1 g, 1.55 mmol) is dissolved in anhydrous DMF (15 mL). 1-Hydroxybenzotriazole (0.24 g, 1.75 mmol) and polyethylene glycol-propionic acid-NHS-ester (1.23 g, 1.75 mmol) are added to the reaction mixture. The mixture is stirred under argon at room temperature for 2 hours after which it is concentrated *in vacuo*. Residual DMF is coevaporated with toluene. The residue is dissolved in dichloromethane (50 mL) and then washed with

an equal volume saturated $NaHCO_3$. The aqueous layer is washed with dichloromethane and the combined organic extracts washed with an equal volume saturated NaCl. The aqueous layer is washed with dichloromethane and the combined organic layers dried over $MgSO_4$ and concentrated. The residue is chromatographed on a silica gel column, eluting with a gradient of 50% ethyl acetate in hexanes to 100% ethyl acetate. The desired product (1.08 g, 58%) eluted with 100% ethyl acetate.

Y. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(Ω-methyl-polyethylene glycol-propionoyl)amino]uridine-3'-O-(2-cyanoethoxy-N,N-diisopropyl)phosphoramidite

5'-O-(Dimethoxytrityl)-2'-O-[hexyl-N-(Ω-methyl-polyethylene glycol-propionoyl)amino]uridine (1.04 grams, 0.87 mmol) is dissolved in dry dichloromethane (20mL). 2-Cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (800 μL, 2.4 mmol) and diisopropylamine tetrazolide (0.090 grams, 0.52 mmol) are added to the mixture, which is stirred under argon for 20 hours. The reaction mixture then is concentrated *in vacuo* and the residue dissolved in dichloromethane (75 mL). The solution is washed with an equal volume of saturated $NaHCO_3$. The aqueous layer is washed with dichloromethane (20 mL) and the combined organic layers washed with an equal volume of saturated NaCl. The aqueous layer is washed with dichloromethane (20 mL) and the combined organic layers dried over $MgSO_4$ and concentrated. The residue is chromatographed on a silica gel column, eluting with a gradient of 50% ethyl acetate in hexanes to 100% ethyl acetate. The desired product elutes with 100% ethyl acetate.

Z. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(Ω-methyl-polyethylene glycol-propionoyl)amino]uridine-3'-O-(succinyl-aminopropyl) controlled pore glass

Succinylated and capped controlled pore glass (CPG) is dried under vacuum for 3 hours immediately before use. Controlled pore glass (0.3 grams) is added to 3 ml anhydrous pyridine in a 50 ml round-bottom flask. DEC (0.12 grams, 0.67 mmol), TEA (25 μl, distilled over CaH_2), DMAP (0.005 grams, mmol) and 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(w-methyl-polyethylene glycol-propionoyl)amino]uridine (0.21 grams, 0.175 mmol) are added under argon and the mixture shaken mechanically for 19 hours. More nucleoside (0.025 grams) is added and the mixture shaken an additional 5.5 hours. Pentachlorophenol (0.045 grams, 0.17 mmol) is added and the mixture shaken 18 hours. CPG is filtered off and washed successively with dichloromethane, triethylamine, and dichloromethane. CPG then is dried under vacuum, suspended in 15 ml piperidine, and shaken 15 minutes. CPG is filtered off, washed thoroughly with dichloromethane, and again dried under vacuum. The extent of loading is determined by spectrophotometric assay of dimethoxytrityl cation in 0.3 M p-toluenesulfonic acid at 498 nm. to be approximately 18 μmol/g.

AA. Preparation of macrocycle derivatized nucleoside

5'-O-(dimethoxytrityl)-2'-O-(hexylamine)uridine is treated as per the procedure of Example 3 with the macrocycle 4-{1,4,8,11-tetraza-[tri-(trifluoroacetyl)cyclotetradec-1-yl]}methyl benzoic acid-N-hydroxy succinimide ester (prepared according to Simon Jones *et. al.* (*Bioconjugate Chem.* 1991, *2*, 416) to yield the product.

AB. Preparation of macrocycle derivatized uridine phosphoramidite

The nucleoside product of Example 4(AA) is treated as per the procedure of Example 4(B) to yield the product.

AC. Preparation of CPG derivatized with macrocycle derivatized nucleoside

The nucleoside product of Example 4(AA) is treated as per the procedure of Example 4(C) to yield the product.

AD. Preparation of 5'-O-(dimethoxyltrityl)-2'-O-(hexyl-N-(folate)amino)uridine

5'-O-(Dimethoxytrityl)-2'-O-(hexylamine)uridine is treated as per the procedure of Example 4(A) with folic acid pentafluorophenyl ester (protected with an isobutyryl protecting group) to yield the product.

AE. Preparation of 5'-O-(dimethoxyltrityl)-2'-O-[hexyl-N-(folate)amino]uridine-3'-O-(2-cyanoethoxy-N,N-diisopropyl)phosphoramidite

The nucleoside product of Example 4(AB) is treated as per the procedure of Example 4(B) to yield the product.

AF. Preparation of CPG derivatized with 5'-O-(dimethoxyltrityl)-2'-O-(hexyl-N-(folate)amino)-uridine nucleoside

The nucleoside product of Example 4(AE) is treated as per the procedure of Example 4(C) to yield the product.

AG. Preparation of 5'-O-(dimethoxytrityl)-2'-O-{hexyl-N-[2-methoxy-6-chloro-9(Ω-amino-caproyl)acridine]amino}uridine

6,9-Dichloro-2-methoxyacridine (Adlrich, 10 g, 36 mmol) and phenol (2.5 g) were placed together on a round-bottom flask with a stirring bar and to this 6-amino-hexanoic acid (9.3 g, 71 mmol) was added and the flask was heated to 100°C (oil bath) for 2 hours. TLC (10% methanol in methylene chloride) showed complete disappearance of starting material. The reaction mixture was cooled and poured into 200 mL of methanol. The product isolates out as a yellow solid (about 10 g). This compound was then converted into its pentafluoro-phenol ester.

5'-O-(Dimethoxytrityl)-2'-(hexylamino)uridine (0.5g, 0.78 mmol) is dissolved in anhydrous DMF (15 mL). 1-Hydroxybenzotriazole (0.16 grams, 1.17 mmol) and 2-methoxy-6-chloro-9-(Ω-caproyl-amino) acridine pentafluorophenyl ester (0.53 grams, 1.17 mmol) are added to the reaction mixture. The mixture is stirred under argon at room temperature for 2 h, after which it is concentrated *in vacuo*. Residual DMF is coevaporated with toluene. The residue is dissolved in dichloromethane (50 mL) and washed with an equal volume saturated $NaHCO_3$. The aqueous layer is washed with dichloromethane and the combined organic extracts washed with an equal volume

saturated NaCl. The aqueous layer is washed with dichloromethane and the combined organic layers dried over $MgSO_4$ and concentrated. The residue is chromatographed on a silica gel column, eluting with a gradient of 50% ethyl acetate in hexanes to 100% ethyl acetate. The desired product elutes with 100% ethyl acetate.

AH. Preparation of 5'-O-(dimethoxytrityl)-2'-O-{hexyl-N-[2-methoxy-6-chloro-9-(Ω-amino-caproyl)acridine]amino}uridine-3'-O-(2-cyanoethyl-N-N-diisopropyl) phosphoramidite

5'-O-Dimethoxytrityl-2'-O-{hexyl-N-[2-methoxy-6-chloro-9-(w-amino-caproyl)acridine]amino}uridine (0.80 grams, 0.77 mmol) is dissolved in dry dichloromethane (20mL). 2-Cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (800 μL, 2.4 mmol) and diisopropylamine tetrazolide (0.090 grams, 0.52 mmol) are added to the mixture, which is stirred under argon for 20 hours. The reaction mixture is then concentrated *in vacuo* and the residue dissolved in dichloromethane (75 mL). The solution is washed with an equal volume of saturated $NaHCO_3$. The aqueous layer is washed with dichloromethane (20 mL) and the combined organic layers washed with an equal volume of saturated NaCl. The aqueous layer is washed with dichloromethane (20 mL) and the combined organic layers dried over $MgSO_4$ and concentrated. The residue is chromatographed on a silica gel column, eluting with a gradient of 50% ethyl acetate in hexanes to 92% ethyl acetate. The desired product elutes with 100% ethyl acetate.

AI. Preparation of 5'-O-(dimethoxytrityl)-2'-O-{hexyl-N-[2-methoxy-6-chloro-9-(Ω-aminocaproyl)acridine]amino}uridine-3'-O-(succinyl aminopropyl) controlled pore glass

Succinylated and capped controlled pore glass (0.3 grams) is added to 3 ml anhydrous pyridine in a 50 ml round-bottom flask. DEC (0.12 grams, 0.67 mmol), TEA (25 μl, distilled over CaH_2), DMAP (0.005 grams, 0.04 mmol) and 5'-O-dimethoxytrityl-2'-O-{hexyl-N-[2-methoxy-6-chloro-9-(Ω-aminocaproyl)acridine]amino}uridine (0.21 grams, 0.17 mmol) are added under argon and the mixture shaken mechanically for 19 hours. More nucleoside (0.025 grams) is added and the mixture shaken an additional 5.5 hours. Pentachlorophenol (0.045 grams, 0.17 mmol) is added and the mixture shaken 18 hours. CPG is filtered off and washed successively with dichloromethane, triethylamine, and dichloromethane. CPG is then dried under vacuum, suspended in 15 ml piperidine and shaken 15 minutes. CPG is filtered off, washed thoroughly with dichloromethane and again dried under vacuum. The extent of loading is determined by spectrophotometric assay of dimethoxytrityl cation in 0.3 M p-toluenesulfonic acid at 498 nm. to be approximately 27 μmol/g.

AJ. Preparation of 5'-O-(dimethoxytrityl)-2'-O-[(hexyl-N,N-dimethyl)amino]uridine

5'-O-(dimethoxytrityl)-2'-O-(hexylamino)uridine (0.19 grams, 0.29 mmol) is dissolved in 4 ml methanol. Sodium acetate pH 4.0 (2 ml), sodium cyanoborohydride (0.02 grams, 0.3 mmol) and 37% formaldehyde in water (300 μl) are added to the reaction mixture, which is stirred 2 hours, after which it is concentrated *in vacuo*. The

residue is dissolved in dichloromethane (50 mL) and washed with an equal volume saturated $NaHCO_3$. The aqueous layer is washed with dichloromethane and the combined organic extracts washed with an equal volume saturated NaCl. The aqueous layer is washed with dichloromethane and the combined organic layers dried over $MgSO_4$ and concentrated. The residue is chromatographed on a silica gel column, eluting with a gradient of 50% ethyl acetate in hexanes to 100% ethyl acetate. The desired product (0.15 grams, 80%) elutes with 10% Methanol-90% ethyl acetate.

EXAMPLE 5: OLIGONUCLEOTIDES HAVING A 3'-ALKYLAMINO GROUP

3'-O-Hexyl-(N-phthalimido)-aminouridine-CPG, *i.e.* the 5'-O-dimethoxytrityl-3'-O-[hexyl-(Ω-N-phthalimido amino)]-uridine-2'-O-(succinyl-aminopropyl) controlled pore glass from Example 4(C), was used to synthesize the following oligonucleotides:

Oligomer 49: 5'-GACU*
Oligomer 50: 5'-GCC-TTT-CGC-GAC-CCA-ACA-CU (SEQ ID NO:67)
Oligomer 51: 5'-GCC-TTT-CGC-GAC-CCA-ACA-CU* (SEQ ID NO:13),

wherein "*" denotes the 3'-O hexylamino-modified nucleoside. Standard commercial phosphoramidites were used with the synthesis cycle times specified by the manufacturer in a 380B ABI instrument (Applied Biosystems).

Oligomer 49 was used for structural proof of 3'-O-alkylamine-bearing oligonucleotides at the 3'-terminal end. It showed the expected three ^{31}P NMR signals (-0.5

ppm, -0.25 ppm, -0,2 ppm) and seven lines in the trace aromatic base region in ^1H NMR its spectrum.

Oligomer 51 was used to demonstrate the nuclease resistance offered by this the alkylamino group and also for further conjugation. The oligomer was treated with pyrene-butyric acid-N-hydroxy succinimide ester in 0.2 M $NaHCO_3$ buffer/DMF. The product, Conjugate 1, was purified by HPLC and size exclusion methods. HPLC retention times (eluting with a gradient of 5% CH_3CN for 10 minutes then 5%-40% CH_3CN for 50 minutes) were as follows: Oligomer 50, 25.99 min.; Oligomer 51, 25.91 min.; and Conjugate 1, 49.35 min.

The nuclease stability of Oligomer 51 and the conjugate were tested against Oligomer 50 in HeLa cytoplasmic/nuclear extracts. The cell extract was diluted 1.4 times. The final concentration of oligonucleotide was 20 μM. The half lives of the oligonucleotides were as follows: Oligomer 50, 1.0 hrs.; Oligomer 51, 3.5 hrs.; and Conjugate 1, 3.6 hrs.

The half life of phosphodiester Oligomer 50 increased 3-4 times by simple modification at the 3'-end with the hexylamino group by itself, an attribute that was unaffected by further conjugation.

EXAMPLE 6: 2'-O-MODIFIED OLIGONUCLEOTIDES

A. GCGTGU*CTGCG where U* is hexyl-(Ω-N-phthalimido)amino]-uridine

The phosphoramidite from Example 4(B), 5'-O-(dimethoxytrityl)-2'-O-[hexyl-(Ω-N-phthalimido)amino]-uridine-3'-O-[(2-cyanoethyl)-N,N-diisopropyl]phosphoramidite was made as a 0.2 M solution in anhydrous CH_3CN and used to synthesize the following

oligonucleotides in an ABI DNA synthesizer, model 380 B. During the modified amidite coupling, the reaction time was increased to 10 minutes. A coupling efficiency of approximately 90% was observed. After deprotection with concentrated ammonium hydroxide (55°C, 16 hours) the following oligonucleotides were purified by reverse phase HPLC and desalting column (Sephadex G-25):

Oligomer 52: 5'-GCG-TGU*-CTG-CG (SEQ ID NO:14); and

Oligomer 53: 5'-GAU*CT.

B. GCGTGTU'CTGCG where U' is 2'-O-[hexyl-N-(1-pyrene-propyl-carbonyl)amino uridine, Conjugate 2 (Oligomer 52 - pyrene butyrate conjugate

To 20 O.D. of Oligomer 52 in 200 μL of 0.2 M $NaHCO_3$ buffer, 5 ml of pyrene-butyric acid-N-hydroxy succinimide ester in an Eppendorf tube was added followed by 200 μL of DMF. The tube was shaken overnight. The reaction was purified by size exclusion and HPLC to yield 18 O.D. of product.

C. GCGTGTU"CTGCG where U" is 2'-O-[6-bromoacetymido-hex-1yl]-uridine, Conjugate 3 (Oligomer 52 - bromoacetate conjugate)

To 12 O.D. of Oligomer 52 in 100 μL of 0.2 M $NaHCO_3$ buffer, 2 mg bromoacetic acid-NHS ester (N-hydroxy succinimidyl bromoacetate) was added. After leaving the reaction to stand overnight, it was purified by size exclusion and HPLC to yield 7.5 O.D. of product.

D. GCGTGTU^CTGCG where U^ is 2'-O-[hexyl-N-(polyethylene glycol)-propionoyl]amino uridine, Conjugate 4 (Oligomer 52 - PEG conjugate)

[redacted]

To 24 O.D. of Oligomer 52 in 200 μL of 0.2 M $NaHCO_3$ buffer, 20 mg of Polyethylene glycol propionic acid-N-hydroxy succinimide ester was added. The reaction was mechanically shaken overnight and purified by Sephadex G-25 size exclusion and chromatography to yield 22 O.D. of product.

HPLC retention times (eluting with a gradient of 5% CH_3CN for 10 minutes then 5%-40% CH_3CN for 50 minutes in a C-18 Delta-Pak reverse phase column) were as follows: Oligomer 52, 24.05 min.; Conjugate 2, 40.80 min.; Conjugate 3, 26.04 min.; and Conjugate 4, 55.58 min.

Changes in T_m due to pyrene conjugation were evaluated against both DNA and RNA. T_m was measured in 100 mM Na^+, 10 nM phosphate, 0.1 mM EDTA, pH 7 at 4 μM strand concentration.

The results were as follows:

TABLE 5:
MELTING TEMPERATURE OF THE HYBRIDIZATION COMPLEX OF THE OLIGONUCLEOTIDE AND ITS COMPLEMENTARY STRAND

	T_m v. DNA (°C)	T_m v. RNA (°C)
Oligomer 52	50.9	55.5
Conjugate 2	55.3	55.5
Δ_{os2-c2}	(4.4)	(0.0)

The values in parentheses are changes in T_m compared to amino linker in oligomer 52 as a control.

EXAMPLE 7: OLIGONUCLEOTIDE SYNTHESIS USING 2'-*O*-HEXYLAMINOFUNCTIONALIZED URIDINE PHOSPHORAMIDITES

A. 2'-*O*-hexylamino(pyrenebutyrate)uridine phosphoramidite

The amidite 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(1-pyrene propyl carbonyl)amino]uridine-3'-O-(2-cyanoethyl-N,N-diisopropyl) phosphoramidite (0.2M in anhydrous acetonitrile) was used to synthesize the following oligomers, both for NMR studies:

Oligomer 54: 5'-GA**U***CT, and

Oligomer 55: 5'-GCC-G**U***G-TCG,

where **U*** is a 2'-*O*-modified phosphoramidite.

These oligomers were purified trityl-on reverse-phase HPLC, detritylated in 80% acetic acid for one hour and then repurified by RP-HPLC and desalted by size-exclusion chromatography. NMR analysis showed the presence of pyrene peaks.

B. 2'-*O*-hexylamino(dinitrophenyl)uridine phosphoramidite

The amidite 5'-O-(dimethoxytrityl)-2'-O-[hexyl-N-(2,4-dinitrophenyl)amino]uridine-3'-O-(2-cyanoethyl-N,N,-diisopropyl)phosphoramidite (0.18 M in anhydrous acetonitrile) was used to synthesize the oligonucleotides, Oligomers 56 to 63. All are analogues of an ICAM antisense sequence. These oligomers were purified trityl-on by RP-HPLC (Waters Delta-Pak C_{18} column, 300 Å, 7.8 mm x 30 cm, linear 50-min gradient of 5-60% acetonitrile in 0.05 M TEAA pH 7.3), detritylated in 80% acetic acid for one hour and then purified by RP-

HPLC and desalted by size-exclusion chromatography. Data are summarized below in Table 6:

TABLE 6:
HPLC RETENTION TIMES OF OLIGONUCLEOTIDES

	Backbone	Total (O.D.)	Retention Time (min.)
Oligomer 56: GAU*CT	P=O	40	39.16
Oligomer 57 (SEQ ID NO:15): U*GG-GAG-CCA-TAG-CGA-GGC#	P=S	64	39.19
Oligomer 58 (SEQ ID NO:68): U*GG-GAG-CCA-TAG-CGA-GGC	P=S	45	39.21
Oligomer 59 (SEQ ID NO:68): U*GG GAG CCA TAG CGA GGC	P=O	60	37.68
Oligomer 60 (SEQ ID NO:5): U*GG GAG CCA U*AG CGA GGC	P=O	69	38.58
Oligomer 61 (SEQ ID NO:16): TGG GAG CCA U*AG CGA GGC	P=O	86	32.38
Oligomer 62 (SEQ ID NO:17): U***CT GAG TAG CAG AGG AGC TC#**	P=O	34	35.76
Oligomer 63 (SEQ ID NO:69): U*GG GAG CCA U*AG CGA GGC#	P=S	72	43.37

#=Non-nucleoside 6-carbon amino linker (Glen Research) and **Bold** indicates nucleotides having 2'-O-methyl substitutions

C. Oligonucleotide synthesis using 2'-*O*-[hexylamino-(cholesterol)]uridine phosphoramidite

The amidite 5'-O-dimethoxytrityl-2'-O-[hexyl-N-(3-oxycarbonyl-cholesteryl)amino]uridine-3'-O-[2-cyanoethyl-N,N,-diisopropyl]-phosphoramidite (0.2M in anhydrous acetonitrile/dichloromethane 2:1 v/v) was used to synthesize Oligomers 67-74. These oligomers are purified

trityl-on by reverse-phase HPLC (Waters Delta-Pak C_{18}, 300Å, 7.8 mm x 30 cm, linear 55-min gradient of 5-80% acetonitrile in 0.05 M TEAA pH 7.3), detritylated in 80% acetic acid for one hour and then repurified by RP-HPLC and desalted by size-exclusion chromatography. Data are summarized below in Table 7.

TABLE 7:
HPLC RETENTION TIMES OF OLIGONUCLEOTIDES

	Backbone	Target (use)	Retention Time (min.)
Oligomer 67: GA**U***CT	P=O	(NMR)	52.73
Oligomer 68 (SEQ ID NO:68): **U***GG-GAG-CCA-TAG-CGA-GGC	P=O	ICAM	49.64
Oligomer 69 (SEQ ID NO:18): **U***GC-CCA-AGC-TGG-CAT-CCG-TCA	P=S	ICAM	51.98
Oligomer 70 (SEQ ID NO:19): **U***GC-GTT-TGC-TCT-TCT-TCT-TGC-G	P=S	CMV	52.57
Oligomer 71 (SEQ ID NO:20): **U***GC-ATC-CCC-CAG-GCC-ACC-AT	P=S	mseICAM	53.24
Oligomer 72 (SEQ ID NO:21): **U***CC-CGC-CTG-TGA-CAT-GCA-TT	P=S	Raf	53.95
Oligomer 73 (SEQ ID NO:22): G**U***T-CTC-GCT-GGT-GAG-TTT-CA	P=S	PKCα	51.04

Oligomer 74 (SEQ ID NO:23): P=S ICAM 52.75

Fl-UU*GG-GAG-CCA-TAG-CGA-GGC

(Fl-U = U 2'-modified with fluorescein; see Example 8(A))

D. Synthesis of oligonucleotides using 2'-*O*-[hexylamino-(fluorescein)] amidite

The amidite 5'-O-dimethoxytrityl-2'-O-[hexyl-N-(5-thiocarbonyl-3,6-dipivolyl fluorescein)amino]uridine-3'-O-(cyanoethyl-N,N-diisopropyl phosphoramidite) (0.2 M in anhydrous acetonitrile) was used to synthesize Oligomer 74 (above) and Oligomers 75-82 on a 1 x 10^5 (Oligomer 75) or 1 x 10^2 (remaining Oligomers) μmol scale. These oligomers are purified trityl-on by reverse phase HPLC (Waters Delta-Pak C_{18}, 300Å, 7.8 mm x 30 cm, linear gradient of acetonitrile in 0.05 M TEAA pH 7.3), detritylated in 80% acetic acid for one hour and then repurified by RP-HPLC and desalted by size-exclusion chromatography.

TABLE 8:
STRUCTURES OF OLIGOMERS 75 TO 82

	Backbone	Target
Oligomer 75:		
GA**U***CT	P=O	(NMR)
Oligomer 76 (SEQ ID NO:68):		
U*GG-GAG-CCA-TAG-CGA-GGC	P=O	ICAM
Oligomer 77 (SEQ ID NO:73):		
U*GC-CCA-AGC-TGG-CAT-CCG-TCA	P=S	ICAM
33		
Oligomer 78 (SEQ ID NO:70):		
U*GC-CCA-AGC-TGG-CAT-CCG-TCA#	P=S	ICAM
Oligomer 79 (SEQ ID NO:19):		
U*GC-GTT-TGC-TCT-TCT-TCT-TGC-G	P=S	CMV
Oligomer 80 (SEQ ID NO:20):		
U*GC-ATC-CCC-CAG-GCC-ACC-AT	P=S	mseICAM
Oligomer 81 (SEQ ID NO:71):		
U*GC-ATC-CCC-CAG-GCC-ACC-A**(U-CPG)**	P=S	mseICAM,
where **(U-CPG)**=2'-*O*-hexylphthalimido U **6**		
Oligomer 82 (SEQ ID NO:22):		
G**U***T-CTC-GCT-GGT-GAG-TTT-CA	P=S	PKC,

where **U*** is U modified with fluorescein.

EXAMPLE 8: PREPARATION OF DERIVATIZED THYMIDINE PHOSPHORAMIDITES

A. 3-Benzyloxymethyl-3'-benzyloxymethyl-5'-O-tert-butyldiphenyl silylthymidine

To a mechanically stirred solution of 5'-O-tertbutyldiphenylsilylthymidine (170 g, 350 mmol) and diisopropylethylamine (200 g, 1547 mmol) in methylene chloride (1000 ml) was added dropwise benzyl chloromethylether (171 g, 1092 mmol). Upon completion of a mild exotherm, the reaction was heated to 40°C for 16 h. Whereupon the reaction was washed with cold 5% HCl, H_2O, sat. $NaHCO_3$, dried ($MgSO_4$) and concentrated *in vacuo*. The resulting oil was chromatographed on silica gel (EtOAc/hexane, 8/2) to afford the product as a viscous oil, 251 g (71%). ^1H NMR($CDCl_3$) 1.09 (s, 9H, $(CH_3)_3$), 1.60 (s, 3H, C5-CH_3), 2.05 (ddd, 1H, C2'b), 2.52 (ddd, 1H, C2'a), 3.81 (dd, 1H, C5'HH), 3.94 (dd, 1H, C5'HH), 4.08 (m, 1H, C4'H), 4.5 (m, 1H, C3'H), 4.61 (s, 2H, OCH_2Ph), 4.72 (s, 2H, OCH_2Ph), 4.80 (s, 2H, OCH_2O), 5.51 (s, 2H, NCH_2O), 6.39 (m, 1H, C1'H), 7.26-7.5 (m, 21H, CH=,ArH). Anal. Calcd. for $C_{42}H_{48}N_2O_7Si$: C, 69.97; H, 6.71; N, 3.89. Found: C, 69.81; H, 6.42; N, 3.91.

B. 3-Benzyloxymethyl-3'-benzyloxymethylthymidine

A solution of 3-benzyloxymethyl-3'-benzyloxymethyl-5'-O-tert-butyldiphenylsilylthymidine (20 g, 28 mmol) in THF (200 ml) was treated with *tert*-butyl ammonium fluoride 1M/THF (40 ml, 40 mmol) at room temperature for 16 hrs. The solution was concentrated *in vacuo* and the resulting oil chromatographed on silica gel (EtOAc/hexane, 7/3→8/2) to afford the product, 10 g (75%). m.p. 83-84°C; ^1H NMR ($CDCl_3$), 1.92 (s, 3H, C5-

CH_3), 2.20-2.50 (m, 3H, C2'H, C5'OH), 3.73 (dd, 1H, C5'HH), 3.89 (dd, 1H, C5'HH), 4.09 (m, 1H, C4'H), 4.49(m,1 H, C3'H) 4.62 (s, 2H, OCH_2Ph), 4.70 (s, 2H, OCH_2Ph), 4.81 (s, 2H, OCH_2O), 5.49 (s,2H, NCH_2O), 6.19 (t, 1H, C1'H), 7.26-7.37 (m, 5H, CH=,ArH). Anal. Calcd. for $C_{26}H_{30}N_2O_7$: C, 64.94; H, 6.26; N, 5.75. Found: C, 64.71; H, 6.27; N, 5.81.

C. 3-Benzyloxymethyl-3'-benzyloxymethylthymidine-5'-aldehyde

A solution of 3-benzyloxymethyl-3'-benzyloxymethylthymidine (14.5 g, 30 mmol) in DMSO (200 ml) was treated with DCC (33 g, 160 mmol) and phosphoric acid 85% (2.0 g) for 16h. The reaction mixture was filtered and concentrated *in vacuo*. The resultant oil was chromatographed on silica gel (EtOAc/hexane, 7/3) to afford the product as a viscous oil, 11 g (76%). ^{1}H NMR ($CDCl_3$) 1.92 (s, 3H, C5-CH_3), 2.20-2.52 (m, 2H, C2'H), 4.09 (m, 1H, C4'H), 4.49 (m, 1H, C3'H), 4.62 (s, 2H, OCH_2Ph), 4.70 (s, 2H, OCH_2Ph), 4.80 (s, 2H, OCH_2O), 5.50 (s, 2H, NCH_2O), 6.28 (t, 1H, C1'H) 7.24-7.51 (m, 11H, ArH, CH=), 9.65 (s, 1H, CHO). Anal. Calcd. for $C_{26}H_{28}N_2O_7$: C, 64.99; H, 5.87; N, 5.83. Found: C, 64.68; H, 5.95; N, 6.01.

D. 3-Benzyloxymethyl-3'-O-benzyloxymethyl-5'-deoxy-5'-N-(octa-decylamino)thymidine

A suspension of 3-benzyloxymethyl-3'-benzyloxymethylthymidine-5'-aldehyde (11 g, 23 mmol) and molecular sieve-4a (12 g) in tetrahydrofuran (250 ml) was treated with octadecylamine (8 g, 30 mmol) for 16 hrs at room temperature. The mixture was then treated with sodium cyanoborohydride (95%, 2.2 g, 33 mmol) and let stir an additional 16 hrs. The reaction mixture was

filtered, concentrated *in vacuo*, partitioned between EtOAc/H_2O, separated, dried and reconcentrated in vacuo. The resultant gum was chromatographed on silica gel to afford a white powder. Recrystallization (MeOH) yielded the product, 3.8 g (23%). m.p. 60-62°C, ^{1}NMR ($CDCl_3$) .88 (m, 3H, CH_3), 1.22-1.51 (m, 35H, CH_2), 1.93 (s, 3H, C5-CH_3), 2.07 (ddd, 1H, C2'a), 2.46 (ddd, 1H, C2'b), 2.51-2.94 (m, 4h, CH_2NH, C5'H), 4.07 (m, 1H, C4'H), 4.28 (m, 1H, C3'H), 4.62 (s, 2H, OCH_2Ph), 4.70 (s, 2H, OCH_2Ph), 4.80 (s, 2H, OCH_2O), 5.50 (s, 2H, NCH_2O), 6.28 (t, 1H, C1'H), 7.25-7.40 (m, 11H, CH=,ArH). Anal. Calcd. for $C_{44}H_{65}N_3O_6$: C, 72.19; H, 8.95; N, 5.74. Found: C, 71.88; H, 8.72; N, 6.01.

E. 3-Benzyloxymethyl-3'-O-benzyloxymethyl-5'-deoxy-5'-N-(octa-decylaminotrifluoroacetyl)thymidine

To a solution of 3-benzyloxymethyl-3'-O-benzyloxymethyl-5'-deoxy-5'-N-(octadecylamino)thymidine (5.8 g, 79 mmol) and TEA (4.0 ml, 28 mmol) in CH_2CH_2 (150 ml) was added trifluoroacetic anhydride (1.2 ml, 85 mmol). After 2h, TLC indicated completeness of reaction. The reaction was concentrated *in vacuo* <40°C and coevaporated with MeOH (2x 25 ml). Chromatography on silica gel (EtOAc/hexane, 1/1) afforded the product, 6.4 g (98%). ^{1}H NMR ($CDCl_3$) .88 (m, 3H, CH_3), 1.25 (m, 32H, CH_2), 1.55 (m, 2H, CH_2CH_2NH), 1.93 (s, 3H, C5-CH_3), 2.10-2.51 (m, 4H, C2'H, CH_2NH), 3.22-3.82 (m, 2H, C5'H), 4.21 (m, 2H, C3'H, C4'H), 4.63 (s, 2H, OCH_2Ph), 4.70 (s, 2H, OCH_2Ph), 4.80 (s, 2H, OCH_2O), 5.50 (s, 2H, NCH_2O), 6.27 (t, 1H, C1'H), 7.23-7.41 (m, 11H, ArH); ^{19}F NMR ($CDCl_3$) -74.68, (DMSO-d_6) -69.36. Anal. Calcd. for $C_{46}H_{66}F_3N_3O_7$: C, 66.56; H, 8.01; N, 5.06. Found: C, 66.41; H, 7.74; N, 5.29.

[redacted]

F. 5'-Deoxy-5-N-(octadecylaminotrifluoro-acetyl)thymidine

A suspension of 3-benzyloxymethyl-3'-O-benzyloxymethyl-5'-deoxy-5-N-(octadecylaminotrifluoroacetyl)thymidine (5.5 g, 66 mmol) in methanol (250 ml), acetone (35 ml), acetic acid (0.5 ml) and palladium hydroxide/carbon (Pearlman's catalyst, 5.5 g) was hydrogenated in a paar bottle for 48 hrs at 50 psi. The catalyst was filtered off on a celite bed and the celite washed carefully with hot acetone (4 x 200 ml). The filtrates were combined, concentrated *in vacuo* to a solid and recrystallized (MeOH) to afford the product, 3.2 g (82%). m.p. 170-172 °C ^{1}H NMR (DMSO-d_6) 88 (m, 3H, CH_3), 1.23 (m, 32H, CH_2), 1.55 (m, 2H, CH_2CH_2NH), 1.80 (s, 3H, C5-CH_3), 2.07 (ddd, 1H, C2'a), 2.45 (ddd, 1H, C2'b), 3.30-3.87 (m, 6H, C2'H, CH_2CH_2NH, C5'CH_2), 3.96 (m, 1H, C4'H), 4.15 (m, 1H, C3'H), 5.20 (m, 1H, C3'OH), 6.18 (t, 1H, C1'H), (20°C) 7.50 (s, 1H, CH=) and 7.55 (s, 1H ,CH=), (90°C) 7.40 (s, 1H, CH=), 11.31 (s, 1H, ArNH), ^{19}F NMR (DMSO) -69.2. Anal. Calcd. for $C_{30}H_{50}N_3O_5F_3$: C, 61.10; H, 8.54; N, 7.12. Found: C, 60.93; H, 8.51; N, 7.34.

G. 5'-deoxy-5'-N-(octadecylaminotrifluoro-acetyl)thymidine-3'-O-(2-cyanoethyl N,N-diisopropyl)phosphoramidite

A solution of 5'-deoxy-5'-N-(octadecylaminotri-fluoroacetyl)thymidine (5.9g, 10 mmol) in dry THF (1000 ml) was treated with bis-N,N-diisopropylaminocyanoethyl phosphite (8.0 g, mmol) and N,N-diisopropylaminotetrazolide (0.5 g, cat. amount) at rm. temp. for 16 h. The reaction was concentrated in vacuo and the residue was chromatography on silica gel

[redacted]

(hexane/EtOAc, 6/4) to afforded the product as a foam (5.1 g). ^{19}F NMR (CDCl3) -74.65; ^{31}P NMR ($CDCL_3$) 149.63, 149.56.

EXAMPLE 9: SYNTHESIS OF CHOLESTEROL-OLIGONUCLEOTIDE CONJUGATES TARGETED TO *MDR1*

Mammalian cells selected for resistance to certain antitumor drugs often display cross resistance to other apparently unrelated drugs and are thus said to display a multidrug resistant (MDR) phenotype (Bradley *et al.*, *Cancer Metastasis Rev.*, 1994, *13*, 223). One form of MDR is based on overexpression of one or more members of a family of membrane proteins (P-glycoproteins) which serve as ATP driven drug efflux pumps (Bradley *et al.*, *Cancer Metastasis Rev.*, 1994, *13*, 223; Gottesman *et al.*, *J. Biol. Chem.*, 1988, *263*, 12163; Roninson, *Biochem. Pharmacol.*, 1992, *43*, 95). The human MDR (P-glycoprotein) gene family has two members, only one of which (P170, encoded by the MDR1 gene) appears to be responsible for resistance to cytotoxic drugs (Roninson, *Biochem. Pharmacol.*, 1992, *43*, 95). In highly drug-resistant cells, P-glycoprotein message and protein levels can be many times greater than in their drug sensitive counterparts. Although MDR can be modulated by using a variety of agents that competitively inhibit P-glycoprotein mediated antitumor drug efflux (Kaji *et al.*, *Biochem.*, 1994, *33*, 5041), several of these agents have proven less than ideal in clinical trials (Chabner *et al.*, *J. Clin. Oncol.*, 1991, *9*, 4).

A. Synthesis

[redacted]

5'-cholesterol conjugated oligonucleotides to the *MDR1* sequence (Chen *et al.*, *Cell*, 1986, *47*, 381; Genbank accession No. AF016535) were synthesized as follows. Cholesterol-3-carboxyaminohexyl-B-cyanoethyl-N, N-diisiopropyl-phosphoramidite was synthesized according to the procedure reported by MacKellar *et al.* (*Nucl. Acids Res.*, 1992, *20*, 3411). 7.25 grams of this amidite was dissolved in anhydrous dichloromethane to bring the concentration to 0.1 M. Using this solution, ISIS 11073, a 5' cholesterol conjugated version of ISIS 5995, was synthesized by standard phosphoramidite chemistry to make an oligomer with a phosphorothioate backbone. For the cholesterol amidite coupling step, reaction time was extended to 45 minutes. This resulted in 85% coupling for the cholesterol amidite. After standard deprotection, the oligonucleotide-cholesterol conjugate was purified on a C-4 reverse-phase HPLC column (Manoharan *et al.*, *Tetrahedron Lett.*, 1995, *36*, 3651). ISIS 12064, a 5' cholesterol conjugated version of ISIS 10221, was synthesized and purified in the same manner.

Oligonucleotides comprising a 3'-O-alkylamino cholesterol moiety were prepared in the following manner. 3'-*O*-(propylthalimide)-cytidine was prepared essentially according to Examples 75 and 104 of WO 95/06659 (PCT/US94/10131, published March 9, 1995), with the exception that N-(5-bromopentyl)phthalimide was used in place of N-(3-bromopropyl)phthalimide in order to generate 3'-*O*-(pentylphthlamide)-cytidine. From this compound, 5'-O-[dimethoxytrityl]-3'-O-[pentylamino]-cytidine was prepared by refluxing with hydrazine in methanol solvent. The product was purified in a silica column using 98% methanol/ammonium hydroxide 2% solvent. 5'-O-[dimethoxytrityl]-3'-O-[pentylamino]-cytidine (1.5

g) was then treated with cholesterol choloformate (1.1 g) in a mixture of 10 ml pyridine in 30 ml methylene chloride. After overnight stirring another 600 mg of cholesterol chloroformate was added and the reaction mixture was stirred for another four hours. Thin layer chromatography analysis showed reaction products at exocyclic amine and at 3'-O-pentylamine side chain. The desired product, 3'-O-[pentylamino-carbonyl-oxy cholesteryl]-cytidine was obtained as the slower moving product in 43% yield.

600 mg of 3'-O-[pentylamino-carbonyl-oxy cholesteryl]-cytidine was coevaporated with pyridine (2x 10 ml) and then dissolved in 10 ml of anhydrous pyridine. With external cooling using a ice bath, trimethylsilyl chloride (1 ml) was added using a syringe over a period of 5 minutes and the solution was stirred for 30 minutes. Then benzoylchloride (1 ml) was added followed by 10 ml of pyridine. The reaction mixture was stirred at room temperature overnight. 4 ml of water was added with external cooling and after 30 minutes 4 ml of concentrated ammonium hydroxide (30%) was added. Stirring was continued for another 1 hour. The reaction mixture was then evaporated and extracted between methylene chloride and water. The methylene chloride layer was evaporated to give 1.2 g of crude N^4-benzoyl-3-O-[pentylamino carbonyl-oxy-cholesteryl]-cytidine which was then purified in a silica column using 2.5% methanol in chloroform. The product identity and homogeneity were confirmed by ^{13}C and ^{1}H NMR spectral studies.

N^4-benzoyl-3-O-[pentylamino carbonyl-oxy-cholesteryl]-cytidine (450 mg) was added to 2 g of controlled pore glass (CPG, succinylated and capped), and to this mixture 200 mg of dimethylaminopyridine, 1 g of

EDC [1-ethyl-3-dimethylaminopropyl)carbodiimide hydrochloride), 400 μl of triethylamine and 10 ml pyridine were added. The mixture was shaken in a wrist-action shaker overnight. The CPG was then filtered, washed with methylene chloride, methanol, methylene chloride and then ether. Then, 1.5 g of pentochlorophenol, 1 g EDC, 1 ml of triethylamine and 10 ml of pyridine were added to the CPG and the shaking was continued for 16 hours. Then 3 ml of piperidine was added and shaking continued for 5 minutes. The CPG was filtered, washed and dried. 9 mg of the derivatized CPG was treated with 25 ml of 2% dichloroacetic acid in methylene chloride and the loading was determined to be 27.16 μm ol/g using colorimetric assays. ISIS 13328 (GATCC*, where C* indicates the 3'-O-alkylamino cholesterol cytidine residue; used for NMR studies), ISIS 13329, ISIS 13330, ISIS 13331 and ISIS 13332 were synthesized using this CPG. Other 3'-O-alkylamino cholesterol derivatives, and 5'-fluorescein isothiocyanate (FITC) conjugates of the oligonucleotides of the invention are prepared in like manner using the methods disclosed in WO 95/06659 and the above protocols.

TABLE 9
Phosphorothioate oligonucleotides targeted to MDR1

ISIS #	SEQUENCES* (DESCRIPTION)	SEQ ID NO:	TARGET REGION
5990	GAG-CCG-CTA-CTC-GAA-TGA-GC	27	5' Untranslated
5993	GTT-CTG-GCT-TCC-GTT-GCA-CC	28	5' Untranslated
5994	CCC-GGC-CCG-GAT-TGA-CTG-AA	29	5' Untranslated
5995	CCA-TCC-CGA-CCT-CGC-GCT-CC	30	Start codon

[redacted]

ISIS #	SEQUENCES* (DESCRIPTION)	SEQ ID NO:	TARGET REGION
10440	CGG-TCC-CCT-TCA-AGA-TCC-AT	31	Start codon
10441	CCC-CTT-CAA-GAT-CCA-TCC-CG	32	Start codon
10442	CAA-GAT-CCA-TCC-CGA-CCT-CG	33	Start codon
5996	CCT-GGT-CAT-GTC-TTC-CTC-CA	34	ORF** (splice junction)
5997	CTT-TGC-CCA-GAC-AGC-AGC-TG	35	ORF (splice junction)
5998	GTT-CAC-TGG-CGC-TTT-GTT-CC	36	ORF / Stop codon
5999	TGA-ACT-TGA-CTG-AGG-AAA-TG	37	3' Untranslated
6002	CTT-GGA-AGA-GCC-GCT-ACT-CG	38	5' Cap region
6003	GCC-GCT-ACT-CGA-ATG-AGC-GC	39	5' Cap region
6004	GGA-AGA-GCC-GCT-ACT-CGA-AT	40	3' Untranslated
6005	CTC-TGT-TCC-TTT-AAT-TAC-GA	41	3' Untranslated
6006	TCC-ACT-TGA-TGA-TGT-CTC-TC	42	3' Untranslated
6007	CTA-TGA-TTT-CTC-TCC-ACT-TG	43	3' Untranslated
6010	GGC-AGT-CAG-TTA-CAG-TCC-AA	44	3' Untranslated
6011	TTT-TAG-CAA-GGC-AGT-CAG-TT	45	3' Untranslated
6012	TGC-AAA-CAT-TTC-AAT-ACT-TT	46	3' Untranslated
6013	AAG-TTT-AGT-TTT-ATT-ATA-GA	47	3' Untranslated

ISIS #	SEQUENCES* (DESCRIPTION)	SEQ ID NO:	TARGET REGION
10221	CAC-CAC-CCC-CCT-CGC-TGG-TC	48	Scrambled # 5995
10222	CTC-CCG-CAC-ATC-TCC-GCG-CC	49	Scrambled # 5995
11432	GCC-ACC-GTC-TGC-CCA-CTC-TG	50	ORF
11433	GGC-ACG-TGC-AAT-GGC-GAT-CC	51	ORF
11434	CGG-AGC-CGC-TTG-GTG-AGG-AT	52	ORF
11435	AGC-AGC-ATC-ATT-GGC-GAG-CC	53	ORF
11436	CGG-CCA-TGG-CAC-CAA-AGA-CA	54	ORF
11437	TGA-ACT-GAC-TTG-CCC-CAC-GG	55	ORF
11438	GGG-ATG-TCC-GGT-CGG-GTG-GG	56	ORF
11439	TGC-CCA-CCA-GAG-CCA-GCG-TC	57	ORF
11440	ATG-CCC-AGG-TGT-GCT-CGG-AG	58	ORF
11441	GCC-TCC-TTT-GCT-GCC-CTC-AC	59	ORF
11442	TGG-TGG-ACA-GGC-GGT-GAG-CA	60	ORF
10443	2'-O-Methyl analog of # 5995	30	Start codon
11073	5'-Cholesterol analog of # 5995	30	Start codon
12064	5'-Cholesterol analog of # 10221	48	Scrambled control for # 11073
13758	2'-O-Methoxyethoxy analog of # 5995	30	Start codon

[redacted]

ISIS #	SEQUENCES* (DESCRIPTION)	SEQ ID NO:	TARGET REGION
13753	2'-Methoxyethoxy analog of # 10221	48	Scrambled control for # 13758
13755	2'-Methoxyethoxy analog of # 5998	36	ORF / Stop codon
14429	2'-O-Methoxyethoxy CTT-ACC-CGC-TTG-TGT-TGC-TG	63	Scrambled control for # 13755
13756	2'-Methoxyethoxy analog of # 6011	45	3' Untranslated
13757	2'-Methoxyethoxy analog of # 6006	42	3' Untranslated
12065	Analog of # 5995 comprising FITC at 3' end	30	Start codon
13329	Analog of # 5995 comprising 3'-O-pentylamino cholesterol	30	Start codon
13330	Analog of # 5995 w/ 5'-C6 amino linker & 3'-O-pentylamino cholesterol	30	Start codon
13331	Analog of # 5995 comprising 5' FITC & 3'-O-pentylamino cholesterol	30	Start codon
13332	Analog of # 10221 comprising 3'-O-pentylamino cholesterol	48	Scrambled control
13409	Analog of # 5995 comprising 5'-C6 amino linker	30	Start codon
13434	Analog of # 5995 comprising FITC at 5' end	30	Start codon

* From left to right, sequences are written from 5' to 3'. All oligonucleotides contain fully substituted phosphorothioate backbones unless otherwise indicated.
** ORF, open reading frame.

B. Oligonucleotide-mediated inhibition of MDR1 mRNA

NIH 3T3 cells transfected with a plasmid containing the human MDR1 gene (pSK1 MDR) have been previously described (Kane *et al.*, *Gene*, 1989, *84*, 439). These cells have proven to be useful models for the study of multi-drug resistance phenomena. Cells were grown in DMEM media containing 10% fetal bovine serum (FBS) and 60 ng/ml colchicine in an atmosphere of 95% air, 5% CO_2.

In most cases, the multi-drug resistant 3T3 cells were exposed to oligonucleotides administered as a complex with cationic liposomes (Lipofectin^R). However, all studies with cholesterol-conjugated (covalently bound) oligonucleotides were performed in the absence of cationic liposomes. Cells were treated with oligonucleotides according to the following procedure. Cells were grown in 162 mm flasks. When 95% confluency was reached, cells were seeded onto 100 mm dishes at 5 x 10^6/dish in 10 ml of 10% FBS/DMEM and incubated for 24 hours. At this stage, the cells were washed two times with phosphate buffered saline (PBS) and then 8 ml of serum-free medium was added. For phosphorothioate oligonucleotides, 20 ug/ml LIPOFECTIN^R (GIBCO/BRL, Gaithersburg, MD) and various amounts of oligonucleotide were mixed, pre-incubated at room temperature for 30 minutes, and then incubated with the cells at 37°C in a CO_2 incubator for various periods. Similar methods were used for 2'-O-methyl phosphorothioate oligonucleotides.

For treatments with cholesterol-phosphorothioate oligonucleotides, the compounds were simply added to the cells in serum free medium (in the absence of LIPOFECTIN^R) with antibiotics and incubated at 37°C in a CO_2 incubator for various periods. The cytotoxicity of the various treatments used in the oligonucleotide experiments was evaluated in preliminary experiments by using a vital dye assay. Unless otherwise noted, conditions were chosen such that there was usually less than a 10% difference in the number of viable cells in samples treated with oligonucleotides versus control samples maintained in medium alone. The MDR-3T3 cells maintained a high level of viability during extended incubation in serum free medium, although cell division was largely suppressed.

To measure MDR1 mRNA expression by Northern blotting, total cellular RNA was isolated by lysis in 4M guanidinium isothiocyanate followed by a cesium chloride gradient, and the RNA was resolved on 1.2% agarose gels containing 1.2% formaldehyde and transferred to nitrocellulose membranes (Dean *et al., J. Biol. Chem.*, 1994, *269*, 16416). The blots were hybridized with a ^{32}P radiolabeled human MDR1 cDNA probe. The MDR1 cDNA probe was isolated by performing a polymerase chain reaction on the pSK1 MDR plasmid, as described previously (Alahari *et al., Nucl. Acids Res.*, 1993, *21*, 4079). The following oligonucleotide primers were used for PCR:

5'-GGATCTTGAAGGGGACCGCAATGGAGGAGC (SEQ ID NO: 61), and
5'-GTCCAACACTAAAAGCCCCAATTAATACAG (SEQ ID NO: 62).

The resulting fragment was checked on an agarose gel and was radiolabeled with ^{32}P-dCTP using a commercially available random primer labeling kit (Amersham, Arlington

Heights, IL) according to the manufacturer's instructions. The filters were hybridized overnight in hybridization buffer (25 mM KPO_4, pH 7.4; 5x SSC; 5x Denhardt's solution, 100 ug/ml Salmon sperm DNA and 50% formamide) (Alahari *et al.*, *Nucl. Acids Res.*, 1993, *21*, 4079). This was followed by two washes with 1x SSC, 0.1%SDS and two washes with 0.25x SSC, 0.1% SDS. Hybridizing bands were visualized by exposure to X-OMAT AR film and quantitated using a PhosphorImager™ (Molecular Dynamics, Sunnyvale, CA). To confirm equal loading of RNA, the blots were stripped and reprobed with a ^{32}P-labeled beta-actin probe (Clontech, Palo Alto, CA).

1. Identification of an antisense oligonucleotide that specifically reduces MDR1 message expression

RNA isolated from MDR 3T3 cells was probed with a 1.0 kb PCR-based MDR1 probe; this revealed a transcript of 4.4 kb. In initial experiments, the MDR 3T3 cells were exposed to 1.0 μm concentrations of several different antisense oligonucleotides, or control oligonucleotides, in the presence of 20 ug/ml LIPOFECTIN^R for 24 hours. One oligonucleotide, ISIS 5995, which was targeted to a region overlapping the AUG codon, caused about 50% reduction in MDR1 message levels (Table 10). Oligonucleotides ISIS 10221 and ISIS 10222 have the same base composition as ISIS 5995, but are "scrambled" sequences that were used as specificity controls. NIH 3T3 cells transfected with pSK1 MDR plasmid were grown to 90% confluence and treated with oligonucleotide (1 μm) for 24 hours in the presence of Lipofectin^R in serum free medium. Total RNA was isolated and fractionated on agarose formaldehyde gels and blotted onto nitrocellulose membranes. These membranes were probed with a ^{32}P

radiolabeled 1.0 kb MDR1 cDNA, and then stripped and reprobed with a ^{32}P radiolabeled beta-actin cDNA probe to confirm equal loading of RNA, allowing the levels of MDR1 transcripts to be normalized with regard to the beta-actin bands. Transcript levels were quantitated using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale, CA); the values are expressed in arbitrary units (the beta-actin transcripts were essentially constant).

TABLE 10:

MODULATION OF MDR1 mRNA BY ANTISENSE OLIGONUCLEOTIDES

ISIS No.	SEQ ID NO:	MDR1 mRNA Level (arbitrary units)	% Control
None*	----	473,913	100%
LIPOFECTIN®	----	430,435	91%
5990	27	443,478	94%
5995	30	226,087	48%
10221	48	456,522	96%
10222	49	406,522	86%

* Control = untreated cells

This experiment was repeated several times, and the MDR1 and beta-actin bands on non-saturated autoradiograms were compared by laser densitometry. The MDR1/beta-actin ratios for the ISIS 5995 and ISIS 10221 oligonucleotides were 0.49 and 1.01, respectively, indicating specific inhibition of MDR1 message levels by ISIS 5995.

2. Time course of inhibition of MDR1 message levels by oligonucleotide ISIS 5995

[redacted]

In order to evaluate the time course of ISIS 5995-mediated MDR1 modulation, transfected cells were treated with 1 μm ISIS 5995, or 1 μm ISIS 10221, for 24, 48 and 72 hours. MDR1 and beta-actin RNA levels were examined as described above. Maximum specific reduction of MDR1 mRNA was observed after 24 hours treatment of cells with ISIS 5995; longer treatment did not result in lower mRNA levels. With these unmodified oligonucleotides, reduction of MDR1 mRNA levels was attained only when oligonucleotide treatment was performed in serum free medium, and when cationic liposomes were used. This result is consistent with previous observations on antisense actions of phosphorothioate oligonucleotides in cell culture (Bennett *et al.*, *Mol. Pharm.*, 1992, *41*, 1023; Manoharan *et al.*, *Tetrahedron Lett.*, 1995, *36*, 3651). Multiple treatments with ISIS 5995 oligonucleotide did not cause any greater specific reduction in MDR1 message levels than a single treatment, while greater cytotoxicity was observed. The reduction in MDR1 mRNA expression was reversible, since, after 24 hours exposure to ISIS 5995, cells returned to culture medium without oligonucleotide demonstrated normal levels of MDR1 mRNA within 24 hours.

3. Concentration dependence of MDR1 message reduction

Treatment of multi-drug resistant 3T3 cells with oligonucleotide ISIS 5995 resulted in a concentration-dependent inhibition of MDR1 message (Figure 1). Some reduction in message levels was observed with concentrations as low as 100 nM. Maximal specific effects were observed at approximately 1.0 μm; this entailed an approximately 60% reduction in MDR1 message.

Use of higher concentrations of oligonucleotides (5-10 μm) with Lipofectin[R] resulted in greater non-specific effects, i.e., reduced beta-actin message levels and increased cytotoxicity, which were observed with both ISIS 5995 and ISIS 10221 (the scrambled control).

C. Oligonucleotide-mediated inhibition of P-glycoprotein

Transfected MDR NIH 3T3 cells were grown and treated with oligonucleotides as described in Example (9)(B). In order to measure P-glycoprotein expression by Western blotting, cells were seeded in 60 mm dishes at 1.2 x 10^6 per dish and incubated for 24 hours in serum containing medium. The cells were treated with the oligomers for various times in serum free medium as described above. Cells were then extracted in lysis buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 500 mM EGTA, 2 mM PMSF, 1 mM DTT, aprotonin (10 ug/ml), 0.5% Triton-X) and sonicated briefly. The lysate was spun in a microfuge tube for 20 minutes at 4°C and the resulting supernatant was checked for protein content. Equal amounts of protein (usually 20 ug) were mixed with SDS sample buffer and boiled. Protein samples were separated by 8% SDS PAGE and the resolved proteins were electrophoretically transferred onto polyvinyllidene fluoride membranes (Millipore, Bedford, MA). The membranes were blocked (with 3% BSA, 2% non fat dry milk in PBS) and then treated with 2 ug/ml C219 anti-P-glycoprotein antibody (Signet, Dedham, MA). After washing three times with 0.1% Tween-20, the membranes were incubated with rabbit anti-mouse antibody (Cappel- Durham, NC). Immunoreactive proteins were visualized either by ECL (Enhanced Chemiluminescence,

Amersham, Arlington Heights, IL) or with ^{125}I secondary antibodies.

Expression of the P-glycoprotein was reduced upon treatment of the multidrug resistant 3T3 cells with the ISIS 5995 oligomer, consistent with the Northern blot analyses of mRNA levels. In the Western assays, P-glycoprotein was reduced at least 80% by treatment with ISIS 5995 and about 50% by ISIS 10440. In contrast, scrambled control oligomer ISIS 10221 did not reduce P-glycoprotein expression. The decrease in P-glycoprotein expression was minimal after 24 hours, readily detectable by 48 hours, and reached a maximum only after 72 hours exposure. Thus, effects at the protein level lag behind the observed reduction in message levels. This observation is consistent with the fact that the P-glycoprotein is quite stable and normally turns over rather slowly with a $t^{1/2}$ of 48-72 hours (Richert *et al.*, *Biochem.*, 1988, *28*, 7607).

D. Effects of a cholesterol derivative of oligonucleotide 5995 on MDR1 message levels and P-glycoprotein expression

A 5'-cholesterol derivative of ISIS 5995 (ISIS 11073), as well as a 5'- cholesterol derivative of the scrambled control oligonucleotide ISIS 10221 (ISIS 12064), were synthesized and their effects on MDR1 message and P-glycoprotein levels were examined. As is explained below, 3'- cholesterol derivatives were also prepared and tested.

Transfected MDR NIH 3T3 cells were grown and treated with oligonucleotides as described in Example (9)(B). MDR1 mRNA expression was measured by Northern blotting as described in Example (9)(C). In order to measure cell surface P-glycoprotein levels by flow cytometry, cells

were seeded in 60mm dishes at 1.2 x 10^6 /plate in 5 ml of medium, grown for one day in 10% FBS/DMEM, and exposed to the oligomers in serum free medium. After treatment with the oligonucleotides, cells were washed twice in PBS, 0.25 ml of pancreatin was added to remove cells from the plate, and the dispersed cells were resuspended in 10% FBS/DMEM and incubated at 37°C for 2 hours. After the incubation, cells were washed in PBS, and 50 μl of 20 ug/ml MRK16 anti-P-glycoprotein antibody (Kamiya, Thousand Oaks, CA) was added (Ishida *et al.*, *Jpn. J. Cancer Res.*, 1989, *80*, 1006). This mixture was incubated for 45 minutes on ice and cells were washed three times in 10% FBS/PBS. Cells were then incubated for 30 minutes with 20 μl of ten times diluted secondary antibody, a R-phycoerythrin (R-PE) conjugated goat anti-mouse IgG (Sigma, St. Louis, MO). After the incubation, cells were washed two times in 10% FBS/PBS. Finally, the cells were resuspended in 500 μl of PBS. The level of R-PE fluorescence in viable cells (as determined by light scatter) was quantitated using the Cicero software application (Cytomation, Fort Collins, CO) on a Becton Dickinson flow cytometer.

Table 11 shows the effect of cholesterol-conjugated phosphothioate oligonucleotides on MDR1 mRNA expression. MDR-3T3 cells were treated with various concentrations of oligonucleotides ISIS 11073 (5'-cholesterol 5995) or ISIS 12064 (5'-cholesterol 10221; scrambled control) for 24 hours in serum free medium. Total RNA was isolated, fractionated and probed with a MDR1 cDNA fragment; the same blot was stripped and reprobed with a beta-actin probe. The results were quantitated using a PHOSPHORIMAGER™; the ratio of MDR1 to beta-actin message is indicated in the table. As shown in Table 11,

treatment with concentrations of ISIS 11073 (cholesterol 5995) in the 250 nM to 2.5 μm range, resulted in a specific decrease in levels of MDR1 message. It is important to note that at least about 60% inhibition of MDR1 mRNA expression was attained with ISIS 11073 without the use of cationic liposomes.

TABLE 11:
MODULATION OF MDR1 mRNA BY CHOLESTEROL-CONJUGATED ANTISENSE OLIGONUCLEOTIDES

ISIS No.	SEQ ID NO:	Conc.	Ratio of MDR1 mRNA to beta-actin mRNA
None*	----	----	1.00
12064	48	2.5 uM	1.10
11073	30	250 nM	0.48
11073	30	500 nM	0.38
11073	30	1.0 uM	0.45
11073	30	2.5 uM	0.59

* Control = untreated cells

Lane 1, untreated cells; lane 2, cells treated with 2.5 μm 5' ISIS 12064; lanes 3-6, cells treated with ISIS 11073 at concentrations of 250 nM, 500 nM, 1.0 μm and 2.5 μm, respectively.

To observe the effects of the 5'-cholesterol 5995 (ISIS 11073) oligonucleotide on the expression of P-glycoprotein at the cell surface, immunofluorescent staining and flow cytometry were utilized. Treatment of multidrug resistant 3T3 cells with increasing concentrations of ISIS 11073 over the range of 0.5-2.5 um resulted in a progressive reduction in surface expression of P-glycoprotein to about 40% of control levels. Some non-specific reduction of P-glycoprotein expression was also observed with the scrambled control oligomer (5'-cholesterol 10221, ISIS 12064), but the effect of the antisense compound was greater at all concentrations tested. In a parallel experiment, the effect of 1 μm

ISIS 5995 or ISIS 10221 administered with LIPOFECTIN^R on P-glycoprotein surface expression was examined. The test concentrations of antisense (ISIS 5995) or scrambled 5'-cholesterol (ISIS 10221) phosphorothioate oligonucleotides, administered with LIPOFECTIN^R, were less effective than an equivalent concentration of the cholesterol-conjugated analogs. Thus, the cholesterol phosphorothioate antisense compound given alone is at least as effective as the phosphorothioate antisense compound administered with cationic liposomes. Moreover, the cholesterol oligonucleotides showed less experiment-to-experiment variation than did the standard phosphorothioate oligonucleotides when administered with cationic lipids.

To observe the effects of the 3'-cholesterol 5995 oligonucleotide (ISIS 13329) on the expression of P-glycoprotein at the cell surface, immunofluorescent staining and flow cytometry were utilized as described above. Treatment of multidrug resistant 3T3 cells with increasing concentrations of ISIS 13329 over the range of 0.5-2.5 μm resulted in a progressive reduction in surface expression of P-glycoprotein to about 50% of control levels. In a parallel experiment, the effect of 1 μm ISIS 5995 or ISIS 13332 administered with LIPOFECTIN^R on P-glycoprotein surface expression was examined. The test concentrations of antisense (ISIS 5995) or scrambled 3'-cholesterol (ISIS 13332) phosphorothioate oligonucleotides administered with LIPOFECTIN^R, were less effective than an equivalent concentration of the cholesterol-conjugated analogs. Thus, the cholesterol phosphorothioate antisense compound given alone is at least as effective as the phosphorothioate antisense compound administered with cationic liposomes. However,

the cholesterol oligonucleotides showed less experiment-to-experiment variation than did the standard phosphorothioate oligonucleotides when administered with cationic lipids and are thus preferred. A further advantage of the 3'-cholesterol oligonucleotide is stability to 3'-exonucleases.

E. Effects of oligonucleotides on Rh 123 accumulation

Rhodamine 123 is fluorescent dye that is a substrate for P-glycoprotein and is rapidly transported out of multi-drug resistant cells. Thus, Rh 123 uptake can be employed as a simple and convenient way of assessing the impact of various treatments on the multi-drug resistance phenotype. In order to measure Rhodamine 123 uptake by flow cytometry, the procedure described by Twentyman *et al.* (*Eur. J. Cancer*, 1994, *30*, 1360) was followed with minor changes. Briefly, 7.5 x 10^5 cells were seeded on 6 well plates, incubated for one day, and treated with oligomers as described above. Cells were removed with pancreatin and resuspended in 10% FBS/DMEM. Rhodamine 123 (Sigma, St. Louis, MO) was dissolved in water, added to a final concentration of 1.0 ug/ml; 500 μl samples were taken at several points, washed with medium once, and resuspended in 500 μl of media. Viable cells were analyzed for the accumulation of Rhodamine 123 on a Becton Dickinson flow cytometer using Cicero software.

Treatment of multi-drug resistant 3T3 cells with increasing concentrations of ISIS 11073 (cholesterol-conjugated ISIS 5995) resulted in a progressive decrease in P-glycoprotein expression (Figure 2) that paralleled an increase in Rh 123 accumulation (Figure 3). As in the case of P-glycoprotein expression, some non-specific effect was also observed with the scrambled control

oligomer (ISIS 12064; cholesterol-conjugated ISIS 10221); however, the effect of the antisense compound was significantly greater.

EXAMPLE 10: UPTAKE AND INTRACELLULAR DISTRIBUTION OF CHOLESTEROL-CONJUGATED OLIGONUCLEOTIDES

As Example 9 demonstrates, experiments indicated that the 3'-cholesterol derivative of ISIS 5995 (ISIS 13329) had an equivalent effect on P-glycoprotein expression as 5'-cholesterol 5995 (ISIS 11073). To determine the rate of cellular uptake and intracellular distribution of MDR1 antisense oligonucleotides, the following experiments were performed with 5'-FITC, 3'-cholesterol oligonucleotides.

The cellular accumulation of FITC labeled oligonucleotides was quantitated by flow cytometry. The cell uptake and intracellular distribution was visualized on a cell-by-cell basis using digitized fluorescence microscopy, essentially as described previously (Shoji *et al.*, *Nucl. Acids Res.*, 1991, *19*, 5543), except that a confocal microscope system was used. Intracellular fluorescence was visualized by taking optical sections through the cell body; a section approximately half way between the top surface of the cells and the surface of the cover slip was examined in each case. Phase contrast images of the same cells were also obtained. A Nikon Fluor 40/1.3 Oil Ph4DL objective was used, with Comos software controlling a Biorad MRC600 scanner/laser. In digitized images, gain and black level settings were optimized on cells treated with free FITC 5995 oligomer and were unchanged thereafter.

Incubation conditions for the flow cytometry and confocal microscopy experiments were as follows. MDR 3T3

cells were treated with 1 μm ISIS 13331 (5'-FITC, 3'-cholesterol 5995), or with 1 μm ISIS 13434 (5'-FITC 5995) with or without LIPOFECTIN^R, for either 2 hours (Figure 4) or 18 hours (Figure 5) in serum free medium at 37□C. Cells were harvested and the fluorescence profiles were determined using a flow cytometer; light scatter parameters were set so as to exclude non-viable cells. In the case of the 18-hour treatment with LIPOFECTIN^R there were some cells with very high levels (above 10^4 units) of fluorescence; these were accumulated in one channel and are shown as a vertical line at the right hand margin of the plot (Figure 5). Cells plated on glass coverslips were treated as above for 18 hour and then examined by confocal microscopy as described above.

As seen in Figure 4, during a 2-hour incubation period the cholesterol conjugated oligonucleotide (ISIS 13331) was rapidly accumulated by cells, while both free ISIS 13434, and ISIS 13434 complexed with LIPOFECTIN^R, accumulated to a far lesser degree. The cellular accumulation of the 3'-cholesterol conjugated 5995 (ISIS 13331) was approximately 40 fold greater than ISIS 13434 at 2 hours. After overnight incubation (Figure 5), the free ISIS 13434 still displayed significantly less cell accumulation than ISIS 13331. The LIPOFECTIN^R complexed ISIS 13434 displayed substantial, but very heterogeneous, cell uptake after overnight incubation. Based on previous experience with stability of phosphorothioates (Akhtar *et al.*, *Trends in Cell Biology*, 1992, *2*, 139) it is believed that most of the fluorescence accumulated represents FITC-conjugated oligonucleotide rather than free FITC.

Confocal microscopic images essentially confirmed and extended the flow cytometry observations. Very

little intracellular accumulation was seen with ISIS 13434. The cells treated with ISIS 13434 complexed with Lipofectin^{R} showed extensive, but very heterogeneous, cellular uptake, with some cells heavily stained and others essentially blank; there was also a background of LIPOFECTIN^{R} particles plus associated oligonucleotide bound to the cover slip. Some of the cells treated with Lipofectin^{R} showed nuclear accumulation of the fluorescence. The cells treated with ISIS 13331 (5'-FITC, 3'-cholesterol) oligonucleotide uniformly displayed extensive fluorescence in both the cytoplasm and nucleus. These observations demonstrate that the cholesterol conjugation has enhanced the rapidity, amount, and uniformity of cellular uptake of the oligonucleotide, and leads to substantial cytoplasmic and nuclear accumulation.

EXAMPLE 11: EFFECTS OF 3'-CHOLESTEROL, 2'-METHOXYETHOXY GAPMER OLIGONUCLEOTIDES ON P-GLYCOPROTEIN EXPRESSION

A. Effects of 2'-methoxyethoxyoligonucleotides on P-glycoprotein expression

2'-Methoxyethoxy derivatives of ISIS 5995 (ISIS 13758, targeted to the start codon region of MDR1), ISIS 5998 (ISIS 13755, targeted to the stop codon region of MDR1) and ISIS 10221 (ISIS 13753, a scrambled control for ISIS 5995) were prepared and examined for their ability to modulate P-glycoprotein expression. To assay activity, experiments were conducted essentially as described for Figure 2 (Example 9). These oligonucleotides are "chimeric" or "gapped" compounds having 2'-methoxyethoxy modifications on the five 5' and five 3' nucleotides, wherein each methoxyethoxy cytosine

is a 5-methyl cytosine, and phosphorothioate deoxynucleotides in the center ten nucleotides of the molecules. This configuration allows the oligonucleotide to have nuclease-resistant "wings" while retaining a central portion that supports the action of RNase H.

As is shown in Figure 6, the 2'-methoxyethoxy derivative of ISIS 5995 (ISIS 13758) exhibited an enhanced ability to inhibit P-glycoprotein expression (compare with Figure 2). Furthermore, ISIS 13755, the 2'-methoxyethoxy derivative of ISIS 5998, exhibited significant activity in these assays, even though the parent compound had an unpromising effect on MDR1 mRNA expression (Example 9(B)). These results are an example of the enhanced efficacy of oligonucleotide activity that can be achieved by incorporating methoxyethoxy modifications into oligonucleotides. Although not wishing to be bound by any particular theory, these results are believed to demonstrate the enhanced activity resulting from the ability of methoxyethoxy modifications to render oligonucleotides resistant to many nucleases, as well as their ability to increase the hybridization affinity of oligonucleotides to their targeted nucleic acid (*i.e.*, RNA or DNA) molecules.

B. Cholesterol-conjugated 2'-methoxyethoxy oligonucleotides

Cholesterol is conjugated to ISIS 13758 (2'-methoxyethoxy targeted to the translation start codon region), ISIS 13753 (2'-methoxyethoxy scrambled control for ISIS 13758), ISIS 13755 (2'-methoxyethoxy targeted to the translation termination codon region) and ISIS 14429 (2'-methoxyethoxy scrambled control for ISIS 13755) using 3'-O-[pentylamino-carbonyl-oxy-cholesteryl]-cytidine CPG

as described above. Biological assays are conducted as described in Example 9. These phosphorothioate oligonucleotides are chimeric compounds having a 3'-cholesterol (for cellular uptake and nuclease resistance), 2'-methoxyethoxy modifications in their flanks (for better binding to the target nucleic acid and for nuclease resistance), and 2'-deoxy nucleotides in the center 10 nucleotide region (the "gap"). This configuration allows the chimeric oligonucleotide to have nuclease-resistant, high affinity "wings" while retaining an unmodified central "gap" that supports the action of RNase H when the oligonucleotide is bound to a target RNA molecule. Placing the cholesterol moiety at the 3'-terminus of the 3' oligonucleotide ensures resistance to 3' exonucleases, enhances cellular uptake, and leaves the 5'-terminus available for conjugation of additional functional groups.

EXAMPLE 12: PLASMA UPTAKE AND TISSUE DISTRIBUTION OF ACTIVE OLIGONUCLEOTIDES IN MICE

The oligonucleotide Oligomer 71 (SEQ ID NO:20) from Example 7(C) was used as a first test oligonucleotide. This olignucleotides is identified in the figures as ISIS 8005. Further, oligonucleotides of the same sequence were prepared in the same manner. These further oligonucleotides include a phosphorothioate oligonucleotide identified in the figures as ISIS 3082 and an oligonucleotide incorporating a C_{18} alkyl group linked to the 5' position of the nucleotides via a 5' amino group (prepared utilizing the compound of Example 8(G) in the same manner as per the procedure of Example 7(C)) identified in the figures as Isis 9047. The

oligonucleotides were tritiated as per the procedure of Graham *et al.* (*Nuc. Acids Res.*, 1993, *16*, 3737-3743).

A. Animals and Experimental Procedure

For each oligonucleotide studied, twenty male Balb/c mice (Charles River Laboratories, Inc., Wilmington, MA), weighing about 25 gm, were randomly assigned into one of four treatment groups. Following a one-week acclimation, mice received a single tail vein injection of ^{3}H-radiolabeled oligonucleotide (approximately 750 nmoles/kg; ranging from 124-170 μCi/kg) administered in phosphate buffered saline, pH 7.0. The concentration of oligonucleotide in the dosing solution was approximately 60μM. One retro-orbital bleed (at either 0.25, 0.5, 2, or 4 hours post-dose) and a terminal bleed (either 1, 3, 8 or 24 hours post-dose) were collected from each group. The terminal bleed was collected by cardiac puncture following ketamine/xylazine anesthesia. An aliquot of each blood sample was reserved for radioactivity determination and the remaining blood was transferred to an EDTA-coated collection tube and centrifuged to obtain plasma. Urine and feces were collected at intervals (0-4, 4-8 and 8-24 hours) from the group terminated at 24 hours.

At termination, the liver, kidneys, spleen, lungs, heart, brain, sample of skeletal muscle, portion of the small intestine, sample of skin, pancreas, bone (both femurs containing marrow) and two lymph nodes were collected from each mouse and weighed. Feces were weighed, and then homogenized 1:1 with distilled water using a Brinkmann Polytron homogenizer (Westbury, NY). Plasma, tissues, urine and feces homogenate were divided

for the analysis of radioactivity by combustion and for determination of intact oligonucleotide content. All samples were immediately frozen on dry ice after collection and stored at -80°C until analysis.

B. Analysis of Radioactivity in Plasma, Tissue, and Excreta

Plasma and urine samples were weighed directly into scintillation vials and analyzed directly by liquid scintillation counting after the addition of 15 ml of BetaBlend (ICN Biomedicals, Costa Mesa, CA). All other samples (tissues, blood and homogenized feces) were weighed into combustion boats and oxidized in a Biological Materials Oxidizer (Model OX-100; R. J. Harvey Instrument Corp., Hillsdale, NJ). The ^3H_2O was collected in 20 ml of cocktail, composed of 15 ml of BetaBlend and 5 ml of Harvey Tritium Cocktail (R. J. Harvey Instrument Corp., Hillsdale, NJ). The combustion efficiency was determined daily by combustion of samples spiked with a solution of ^3H-mannitol and ranged between 73.9-88.3%. Liquid scintillation counting was performed using a Beckman LS 9800 or LS 6500 Liquid Scintillation System (Beckman Instruments, Fullerton, CA). Samples were counted for 10 minutes with automatic quench correction. Disintegration per minute values were corrected for the efficiency of the combustion process.

C. Analysis of Data

Radioactivity in samples was expressed as disintegrations per minute per gram of sample. These values were divided by the specific activity of the radiolabel to express the data in nanomole-equivalents of

total oligonucleotide per gram of sample, then converted to percent of dose administered per organ or tissue. Assuming a tissue density of 1 gm/ml, the nmole/gram data were converted to a total μM concentration. To calculate the concentration of intact oligonucleotide in plasma, liver or kidney at each time point, the mean total μM concentrations were divided by the percent of intact oligonucleotide in the dosing solution (82-97%), then multiplied by the mean percentage of intact oligonucleotide at each time point as determined by CGE or HPLC. These data was then used for the calculation of tissue half-lives by linear regression and to compare the plasma pharmacokinetics of the different modified oligonucleotides. The pharmacokinetic parameters were determined using PCNONLIN 4.0 (Statistical Consultants, Inc., Apex, NC). After examination of the data, a one-compartment bolus input, first order output model (library model 1) was selected for use.

D. Plasma Uptake and Biodistribution Results

The results of the animal plasma uptake and tissue distribution tests are illustrated graphically in Figures 7 to 10. As is seen in Figure 7, plasma concentration of each of the test oligonucleotides decrease from the initial injection levels to lower levels over the twenty-four hour test period. Plasma concentrations of the two oligonucleotides bearing conjugate groups of the invention were maintained at a higher level for a longer period than were those of the non-conjugate bearing phosphorothioate. All of the test compounds were taken up from the plasma to tissues as is shown in Figures 8, 9 and 10. The two compounds of the invention had different

distribution between the various tissues. Figure 8 shows the tissue distribution of the unconjugated phosphorothioate oligonucleotide, ISIS 3082. Figure 9 shows the tissue distribution of oligonucleotide ISIS 9047 (identical to ISIS 3082 but having a C_{18} amine moiety conjugated to the 5' terminal residue), while Figure 10 shows the tissue distribution of oligonucleotide ISIS 8005 (also derived from ISIS 3082 and having a cholesteryl moiety conjugated to the 5' terminal residue). The distribution of 5'-C_{18} amine antisense oligonucleotide (ISIS 9047) was similar to that of parent compound ISIS 3082 except for an increase in distribution to the liver and a somewhat longer retention in plasma. Both of these attributes were further enhanced in the case of the 5'-cholesteryl antisense oligonucleotide, with the amount of hepatic signal continuing to increase for at least 24 hours after dosing.

E. Modulation of ICAM-1 Expression in the Mammalian Liver by 5'-Cholesterol-Conjugated Antisense Oligonucleotides

The results from the animal plasma uptake and tissue distribution studies indicate that ISIS 8005, a 5'-cholesterol-conjugated antisense oligonucleotide targeted to murine ICAM-1, preferentially accumulates in murine livers following i.v. administration. In order to determine if this preferential accumulation results in enhanced modulation of ICAM-1 in the mammalian liver, the following experiments were done.

Mice were treated with oligonucleotides and examined as follows. First, 10 to 30 mg/kg of oligonucleotide (or a control solution) were administered (i.v.) to each mouse 24 and 2 hours prior to LPS treatment (Figure 11

shows results with 30 mg/kg doses). Then, to induce ICAM-1 expression, bacterial lipopolysaccharide (LPS) was injected into the mice. After 2 hours, the mice were sacrificed, and particular organs, or specific portion thereof, were removed for further study. RNA was prepared from isolated, homogenized tissues essentially according to the guanidinium/CsCl purification method of Chirgwin *et al.* (*Biochemistry*, 979, *18*, 5294), electrophoresed and transferred to nylon membranes for hybridization studies (Northern analysis) according to methods well known in the art. In order to detect mouse ICAM-1 mRNA, a radiolabeled probe was prepared by random oligonucleotide-primed synthesis using a mouse ICAM-1 clone as a template. The blots were stripped and reprobed with a ^{32}P-labeled glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe (Clontech Laboratories, Inc., Palo Alto, CA) in order to confirm equal loading of RNA and to allow the levels of ICAM-1 transcripts to be normalized with regard to the G3PDH signals. Hybridizing bands were visualized by exposure to X-OMAT AR film and quantitated using a PHOSPHORIMAGER™ essentially according to the manufacturer's instructions (Molecular Dynamics, Sunnyvale, CA).

As shown in Figure 11, LPS induced ICAM-1 mRNA levels in the murine liver by at least one order of magnitude relative to untreated animals ("BASAL") whether administered i.v. ("LPS-IV") or i.p. ("LPS-IP"). At the given dosages, in the absence of an uptake facilitator, ISIS 3082 ("3082-30," indicating 30 mg/kg of ISIS 3082) was unable to reverse the LPS-mediated induction of ICAM-1 mRNA in the liver. In contrast, under these conditions, ISIS 8005 ("8005-30"), a 5'-conjugated cholesterol derivative of ISIS 3082, reduced the amount

of hepatic ICAM-1 mRNA by about 50% relative to the amount present in the livers of animals to which LPS, but not oligonucleotide, was administered. A control 5'-cholesterol conjugated scrambled oligonucleotide, ISIS 13293, had no apparent effect on induced ICAM-1 levels.

In similar experiments, the sequence (gene target) specificity of the 5'-cholesterol antisense oligonucleotide ISIS 8005 was examined. ISIS 8005, which is targeted to ICAM-1, had no effect on the levels of mRNA derived from the oncogene c-raf or encoding the cellular adhesion molecule PECAM-1, which is related to, yet distinct from, ICAM-1. As shown in Figure 12, the 5'-cholesterol 3082 analog had no discernable effect on c-raf or PECAM-1 mRNA levels.

To determine if the antisense-mediated inhibition of ICAM-1 mRNA in the mammalian liver is organ-specific, levels of ICAM-1 mRNA in lung samples from the sacrificed animals used in the experiments described above were examined by Northern assays. As is shown in Figure 13, administration of LPS induced ICAM-1 mRNA levels in the lung, albeit to a lesser degree than seen in liver samples. In any event, neither ISIS 3082 nor its 5'-cholesterol conjugate ISIS 8005 was able to reverse the LPS-mediated induction of ICAM-1 to any great degree under these conditions.

Immunohistological sections of isolated liver samples were prepared and stained using primary antibodies specific for ICAM-1 and secondary antibodies conjugated to horse radish peroxidase (HRP). As shown in Figure 14, the cross-section from untreated livers ("Basal") is virtually indistinguishable from livers treated with the ICAM-1 inducer LPS and the 5'-cholesterol conjugated ICAM-1-targeted antisense

oligonucleotide ISIS 8005 (two left panels). In contrast, livers induced by LPS and treated with ISIS 13293, a 5'-cholesterol conjugated scrambled control (5'-GCG-TTG-CTC-TTC-TTC-TTG-CG, SEQ ID NO:64) phosphorothioate oligonucleotide for ISIS 8005 appear quite dark (two panels on right) due to the signal resulting from HRP bound (*via* a series of antibodies) to ICAM-1. These results indicate that levels of the ICAM-1 protein in the mammalian liver can, like the levels of ICAM-1 mRNA in livers, be modulated by a cholesterol-conjugated antisense oligonucleotide in a sequence-specific manner. Moreover, comparison of the overall cellular structure in the untreated ("basal") livers to LPS- and ISIS 8005-treated reveals that there are no gross cellular defects resulting from antisense-mediated reversal of ICAM-1 induction.

For comparison's sake, ISIS 9388, a 3'-cholesterol-conjugated derivative of ISIS 3082 (SEQ ID NO:20), was also prepared. Specifically, ISIS 9388 contains a 3' terminal 3'-O-hexylamino cholesteryl moiety introduced into the oligonucelotide during synthesis using the phosphoramidite from Example 4(B) and other methods disclosed herein. ISIS 9388, and some associated control compounds, were tested for their ability to modulate ICAM-1 mRNA levels in murine livers according to the preceding methods. The results, shown in Figure 15, demonstrate that, like the 5'-cholesterol conjugate (ISIS 8005), the 3'-cholesterol conjugate (ISIS 9388) is able to reduce ICAM-1 mRNA levels by about 50% under these conditions (*i.e.*, a dosage of 10 mg/kg of oligonucleotide).

The results in this Example demonstrate that cholesterol-conjugated antisense oligonucleotides are

preferentially taken up by cultured mammalian cells *in vitro* and targeted to the mammalian liver *in vivo*. Furthermore, the oligonucleotides that are targeted to the liver *in vivo* effect sequence-specific antisense modulation of their target gene in the liver *in vivo*, regardless of the point of attachment of the cholesteryl moiety. Taken together, these findings provide the basis for a method of preferentially targeting a biologically active antisense oligonucleotide to hepatic tissues in mammals, and thereby modulating the expression of a gene in the liver of a mammal, by conjugating the oligonucleotide to a cholesteryl moiety and administering the cholesterol-oligonucleotide conjugate to a mammal.

EXAMPLE 13: UPTAKE OF BIS-CHOLESTERYL-CONJUGATED PHOSPHOROTHIOATE OLIGONUCLEOTIDE

A. Synthesis of ISIS-9389

Starting from the solid support 5'-O-(dimethoxytrityl)-3'-O-[hexyl-N-(3-oxycarbonyl-cholesteryl)amino]uridine-2'-O-succinyl aminopropyl)-controlled pore glass (See Example 4), ISIS-9389, the 3',5'-bis-cholesteryl-conjugated PS-ODN having the same sequence as ISIS-3082 and ISIS-9388, was synthesized using standard deoxynucleotide phosphoramidites according to Example 4 described above. During the oxidation step, Beaucage reagent was used to introduce phosphorothioate linkages. The purification was carried out using a gradient of 5% to 95% CH_3CN. The final product was characterized by HPLC, CGE and mass spectrometry analysis.

B. Preparation of [^{3}H]ISIS-9389

[redacted]

To allow monitoring of its biological fate, ISIS-9389 was radiolabeled with ^3H by heat-catalyzed exchange at the C8 positions of the purine nucleotides as described by Graham et al. (*Nucl. Acids Res.***21**:3737-3743, 1993). [^3H]ISIS-9389 was purified by reverse phase HPLC using a Waters C4 column (5 μM, 300 angstroms, 300x3.9 mm) at a flow rate of 1 ml/min using the following mobile phases: A, 50 mM triethyl ammonium acetate (pH 7.0); B, acetonitrile. After injection of the samples (0.5 ml), the column was eluted for 5 min with 10% B, followed by a gradient of 10-90% B (25 min). Subsequently, the column was eluted for 10 min with 90% B. The retention time of ISIS-9389 under these conditions was approximately 28 min (ISIS-3082 and ISIS-9388: 13 min and 25 min, respectively). The radiolabeled oligonucleotide was precipitated as the Na-salt by adding 10 volumes of 3% (w/v) $NaClO_4$ in acetone as described by Rump et al. (*Bioconj. Chem.* **9**341-349, 1998). The specific radioactivity of [^3H]ISIS-9389 was approximately 50 x 10^6 dpm/mg, and the radiochemical purity >98%.

C. Determination of the stability of ISIS-9389 in rat serum and plasma

[^3H]ISIS-9389 was incubated at 37°C at a concentration of 20 μg/ml with rat serum of EDTA-plasma (4 mM EDTA). After 90 min, aliquots of 200 μl of the incubation mixtures were mixed with an equal volume of extraction buffer (25 mM Tris-HCl buffer, pH 8.0, containing 25 mM EDTA, 100 mm NaCl, 0.5% Nonidet P-40 and 1 mg/ml proteinase K), and incubated for a further 2 h at 56°C. Subsequently, the samples were mixed with 400 μl of phenol/isoamyl alcohol/chloroform (25:1:24; by vol.).

After shaking for 10 min, the phases were separated by centrifugation. The organic phase was washed 4 times with 400 µl of water. The aqueous phases were combined (total extraction efficiency approx. 40%), and dried in a speed-vac concentrator. The residues were dissolved in water, and 30 µg of unlabeled ISIS-9389 was added as marker (final volume 600 µl). An aliquot of 500 µl was subjected to reverse phase HPLC as described above. Fractions of 1 ml were collected and assayed for radioactivity. It was found that after 90 min of incubation of [^{3}H]ISIS-9389 with rat serum or plasma, 95% of the radioactivity eluted at the position of the unlabeled ISIS-9389 marker. As the retention of ISIS-9389 depends of the presence of cholesterol, this indicates that the radiolabeled oligonucleotide still contains both cholesterol moieties, implicating that it was fully intact.

D. Determination of plasma clearance and tissue distribution

Male Wistar rats, weighing between 200 and 350 grams, were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg) and the abdomen was opened. Radiolabeled oligonucleotide, dissolved in PBS, pH 7.4, was injected via the vena penis (2 ml/kg body weight). At the indicated times, blood samples of 0.2-0.3 ml were taken from the inferior vena cava and collected in heparinized tubes. The samples were centrifuged for 2 min at 16,000 x g, and the plasma assayed for radioactivity. Samples containing ^{3}H were counted in a Packard Tri-Carb 1500 liquid scintillation counter without further processing by liquid scintillation spectroscopy, using Emulsifier Safe™ or

Hionic Fluor™ scintillation cocktails (Packard, Downers Grove, IL). The total amount of radioactivity in plasma was calculated using the equation: plasma volume (ml)=[0.0219 x bodyweight(g)] + 2.66.

At the indicated times, liver lobules were tied off and excised, and at the end of the experiment the remainder of the liver was removed. The amount of liver tissue tied off successfully did not exceed 15% of the total liver mass, The amount of radioactivity in the liver at each time point was calculated from the radioactivities and weights of the liver samples. Uptake by extrahepatic tissues was determined by removing the tissues at the end of the experiment, and counting the radioactivity. Tissue samples were processed using a Packard 306 sample oxidizer. Some tissues (e.g. bone) were dissolved in 10 M NaOH at 95°C before counting. Radioactivity in tissues was corrected for radioactivity in plasma present in the tissue at the time of sampling.

The plasma clearance of intravenously injected radiolabeled oligonucleotide was analyzed by a non-linear regression program (GraphPad, ISI Software, San Diego, CA). The data were best fit by a two-compartment model. The distribution volume (V_{dis}) was calculated by extrapolation of the elimination curve to time zero. The half-life of elimination was calculated from the elimination rate constant (k_e) using the formula: $T_{1/2}=0.693/k_e$. The total body clearance (CL) was calculated using the formula $CL=V_{dis} \times k_e$.

The disposition of ISIS-9389 was studied after a bolus injection of the radiolabeled oligonucleotide into rats. The dose, 1 mg/kg body weight, was in the range of doses of ICAM-1-directed antisense oligonucleotides that have been found to be effective in preclinical models and

in patients. The clearance of radioactivity from the blood plasma was followed for 90-180 min. Figure 16 shows the plasma clearance of radioactivity after injection of [^{3}H]ISIS-9389. After an initial rapid distribution phase, radioactivity was cleared from the circulation with a half-life of 23.6±0.3 min. The plasma clearance of ISIS-9389 was followed for 90 min. In Table 12, the pharmacokinetic parameters of ISIS-9389 are compared with those of ISIS-9388 and ISIS-3082. The distribution volumes of the three oligonucleotides were not significantly different. ISIS-9389 was cleared from the circulation at approximately the same rate as the parent compound ISIS-3082, but more rapidly than ISIS-9388.

TABLE 12:
Pharmacokinetic parameters of intravenously injected ISIS-9389, ISIS-3082 and ISIS-9388

	ISIS-3082	ISIS-9388	ISIS-9389
Half-life (min)	23.3±3.8	49.0±2.2	23.6±0.3
Distribution volume (ml/kg)	62.9±9.3	52.6±4.4	59.8±9.9
Total body clearance (ml/min per kg)	1.95±0.25	0.74±0.03	1.75±0.28

At 90 min after injection, when approximately 95% of the injected dose of [^{3}H]ISIS-9389 had been cleared from the circulation, the distribution of the radioactivity over the body was determined. The results are shown in Table 13. The most striking finding is the high amount of radioactivity in the liver.: 83.0±0.8% of the dose, which corresponds to 87.8±0.8% of the cleared amount. The only other tissues that contained significant amounts of radioactivity were the spleen and the bone (marrow).

Liver, spleen and bone(marrow) together accounted for >95% of the clearance of ISIS-9388. Even bulky tissues, like skin and muscle, contained <1% of the dose. Kidneys, which play a prominent role in the disposition of unconjugated PS-ODNs, accumulated only a minor amount of ISIS-9388 (<0.5% of the dose). The kidney uptake of ISIS-9389 is approximately 50 times lower than that of ISIS-3082. The specific uptake of liver, spleen and bone marrow (expressed as relative specific radioactivity which is the % of recovered radioactivity divided by % of recovered weight) is at least one order of magnitude higher than that of any other tissue. Figure 17 compares the tissue distribution of ISIS-9389 with those of ISIS-3082 and ISIS-9388. The liver is the most important tissue in the uptake of all three oligonucleotides, but conjugation with cholesterol significantly enhanced the liver uptake. About 40% of ISIS-3082 was taken up by the liver, whereas the hepatic uptake of the 3'-cholesterol-modified derivative ISIS-9388 was about 70%. Conjugation of a second cholesterol, yielding ISIS-9389, surprisingly resulted in almost exclusive uptake by the liver.

TABLE 13:
Tissue distribution of intravenously injected [^{3}H]ISIS-9389

Tissue	**Radioactivity (% of recovered)**	**Relative specific radioactivity**
Blood plasma	5.3±0.5	
Urine	0.2±0.0	
Liver	83.0±0.8	16.5±0.7
Spleen	3.1±0.3	12.7±0.5
Bone marrow		8.4±0.8
Bone(including	4.3±0.2	0.2±0.0

Tissue	Radioactivity (% of recovered)	Relative specific radioactivity
marrow)		
Kidneys	0.4±0.0	0.4±0.0
Intestines	1.1±0.2	0.1±0.0
Muscles	0.8±0.3	0.0±0.0
Skin	0.5±0.2	0.0±0.0
All other tissues	1.2±0.1	0.1±0.0

E. Determination of ISIS-9389 over liver cell types

The liver contains several actively endocytosing cell types, including parenchymal, endothelial and Kupffer cells. To identify the cell type(s) responsible for the hepatic uptake of ISIS-9389, rats were anesthetized and injected with radiolabeled oligonucleotides as described above. The liver was perfused at 60 min after injection, and parenchymal, Kupffer and endothelial cells were isolated as described by Nagelkerke et al. (*J. Biol. Chem.* **263**:12221-12227, 1983). The cell isolation procedure was performed at a low temperature (8°C) to prevent processing of internalized oligonucleotide. The cell fractions were assayed for radioactivity and protein. Shortly before separation of the cells, a liver lobule was tied off and excised to determine the total liver uptake. The contributions of the various cell types to the total liver uptake was calculated from the uptake per mg of cell protein and the contribution of each cell type to the total liver protein. As found with other ligand, no significant amounts of radioactivity were lost from the cells during the isolation procedure. This was checked in each experiment by comparing the calculated liver

uptake (*i.e.*, the summation of the contributions of the various cell types) with the value actually measured in the liver lobule. The percentage of the administered dose taken up by each cell type was calculated from the contribution of the cells to the total liver uptake and the contribution of the liver to the clearance of ISIS-9389. The intracellular concentrations were calculated from the molecular weight of the oligonucleotide, liver weight (4.3±0.1% of body weight, mean±S.E.M. of 10 determinations), liver density (1.07 mg/ml) and the volumes of the different cellular compartments in the liver. It was assumed that 75% of the cellular volume consists of water.

The results are shown in Table 14. Endothelial cells were the major site of uptake in the liver (51.9%±6.4% of the liver uptake), while parenchymal and Kupffer cells each accounted for about 25% of the liver uptake. When all injected oligonucleotide is cleared from the circulation, 87.7±0.8% of the injected dose was taken up by the liver. It can thus be calculated that parenchymal, Kupffer and endothelial cells accumulate 21.8±6.7%, 20.4±2.2% and 45.5±5.6% of the injected amount of oligonucleotide, respectively. Intracellular concentrations of ISIS-9388 in parenchymal, Kupffer and endothelial cells can be calculated from these data and from the sizes of the three different cellular compartments. Parenchymal and Kupffer cells contain about equal amounts of ISIS-9389; however, parenchymal cells constitute >90% of the cellular mass and Kupffer cells only 2.5%. The concentration of ISIS-9389 in Kupffer cells is therefore much higher than in parenchymal cells (51.5±5.5 μM versus 1.5±0.5 μm).

However, endothelial cells contained the highest concentration of ISIS-9389 (86.1±10.6 μm).

TABLE 14:
Uptake of [^{3}H]ISIS-9389 by liver cell types

Cell type	Contribution to total liver uptake (%)	% of administered dose in cell type	Total cellular load (μM)
Parenchymal	24.9±7.7	21.8±6.7	1.5±0.5
Kupffer	23.3±2.5	20.4±2.2	51.5±5.5
Endothelial	51.9±6.4	45.5±5.6	86.1±10.6

Figure 18 compares the accumulation of ISIS-9389 in the different liver cell types with that of ISIS-3082 and ISIS-9388. Conjugation with cholesterol resulted in higher uptake by all three liver cell types, but uptake by non-parenchymal cells, in particular Kupffer cells, is greater than that of the parenchymal cells.

Bis-cholesterol conjugation of oligonucleotides represents a new therapeutic entity. These conjugated oligonucleotides are almost exclusively taken up by the liver. The highest concentrations were found in the endothelial cells. The selective accumulation of *bis*-cholesteryl-conjugated oligonucleotide in liver endothelial cells is beneficial in targeting genes expressed in these cells. For example, ICAM-1 is upregulated in liver endothelial cells under inflammatory conditions that result in the harmful infiltration of neutrophils into the liver. Systemically administered unconjugated PS-ODNs specific for ICAM-1 reduce the adherence of neutrophils to the cells of the endothelial lining of the liver, and consequently exert a therapeutic

effect (Wong, *Hepatology* **26**:165A, 1997). Higher cellular accumulation of these oligonucleotides due to cholesterol conjugation will result in an improved therapeutic effect. In addition, the reduced uptake by non-target tissues, in particular the kidneys, will minimize side effects since conjugation of two cholesterol moieties reduces accumulation of oligonucleotide in kidneys about 50-fold.

The conjugation of more than two cholesterol moieties to an oligonucleotide for targeting the liver, and endothelial cells of the liver, is also within the scope of the present invention. Conjugation of additional cholesterol moieties to an oligonucleotide can be performed using methods described herein, and other methods well known in the art. In addition, in another embodiment of the invention, two or more of any of the lipophilic moieties disclosed herein, either the same or different, are conjugated to an oligonucleotide for preferentially targeting the liver.

F. Implication of scavenger receptors in the liver uptake of ISIS-9388

The role of scavenger receptors in the hepatic uptake of ISIS-3082 and ISIS-9388 has been previously demonstrated (Bijsterbosch et al., *Nucl. Acids. Res.* **28**:2717-2725, 2000; Bijsterbosch et al., *Nucl. Acids Res.i* ***25****:3290-3296, 1997)*. Endothelial liver cells, and to a lesser extent Kupffer cells, express the type AI/II scavenger receptor (SR-AI/AII), which binds and internalizes a wide variety of polyanionic ligands. Interaction of ligands with SR-AI/AII can be effectively inhibited by polyinosinic acid (poly-I). Polyadenylic acid (poly-A), which has a different ternary structure,

is not inhibitory. To study the possible role of SR-AI/II in the liver uptake of ISIS-9389, rats were preinjected with poly-I or poly-A (Sigma, St. Louis, MO) shortly before injection of [^{3}H]ISIS-9389. The uptake of ISIS-9389 by the liver was substantially inhibited by poly-I (Fig. 19). Poly-A also inhibited the hepatic uptake of ISIS-9388, but was less effective than poly-I. These findings suggest that scavenger receptors play a major role in the hepatic uptake of ISIS-9388.

G. Association of ISIS-9388 and ISIS-3082 with plasma proteins

PS-ODNs bind to plasma proteins which is likely to affect their disposition (Cossum et al., *J. Pharmacol. Exp. Ther.* **267**:1181-1190, 1993). To examine the interaction of ISIS-9389 with plasma proteins, [^{3}H]ISIS-9389 was incubated with rat plasma (at 20 µg/ml; the plasma concentration immediately after intravenous injection). After 30 minutes, the incubation mixture was subjected to size exclusion chromatography on a Superose 6 Precision column (3.2 x 300 mm) equipped with a 50 µl sample loop (Pharmacia, UPPSALA, Sweden). The column was eluted with PBS at a flow rate of 50 µl/min. Fractions of 100 µl were collected and assayed for radioactivity.

The results are shown in Fig. 20. The chromatographic profile of [^{3}H]ISIS-9389 was clearly different from those of [^{3}H]ISIS-9388 and [^{3}H]ISIS-3082. All three oligonucleotides were found to be protein-bound, as no radioactivity was recovered in fractions eluting at 0.90-1.50 ml, which contain high molecular weight plasma proteins. Only a small proportion (15-20%)

was recovered in fractions eluting at 1.50-1.90 ml, which contain the bulk of the plasma proteins.

ISIS-9389 carries two cholesterol residues, which may lead to cross-linking of lipoprotein particles, when ISIS-9389 is present in plasma. To assess the interaction of ISIS-9389 with low density lipoprotein (LDL) and high density lipoprotein (HDL), [^{3}H]ISIS-9389 was incubated with radioiodinated rat LDL and HDL (0.2 mg/ml and 1.0 mg/ml, respectively; the concentrations in rat plasma), and the incubation mixtures were analyzed by size exclusion chromatography. Figure 21 shows that ISIS-9389 associates readily and quantitatively with LDL and HDL. The complexes of ISIS-9389 and both lipoproteins eluted at the same positions as native LDL and HDL, which indicates that ISIS-9389 does not induce cross-linking and aggregation of the lipoprotein particles.